University of Alberta

THE ROLE OF GRANZYME B IN TARGET CELL APOPTOSIS

by

Michael Joseph Pinkoski

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

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R. Chris Bleackley

Charles F.B. Holmes

Stephen A. Rice

Esmond J. Sanders

Jack Gauldie

On this day, the 26 of August in the year 1997.

ABSTRACT

Cytotoxic T lymphocytes (CTL) kill by two mechanisms: granule- and receptor-mediated killing. Both mechanisms appear to share many common features, but differ in the manner in which the death program is initiated. Granule mediated killing involves transfer of cytolytic effector molecules from the CTL into the target. The serine protease, granzyme B, is a major effector that triggers the death program by cleavage and activation of target cell proteases.

At the outset of these studies, there were no known substrates of granzyme B inside target cells. To address the notion that granzyme B enters target cells, binds and cleaves cellular substrates, I developed a protease-linked immunocytochemistry system to visualize granzyme B binding to potential substrates *in situ*. Results from these studies demonstrated that granzyme B binds to target cell proteins in a substrate dependent manner. Application of this assay to track granzyme B also provided insight into the mechanism of entry of granzyme B into target cells.

When it became evident that regulated trafficking of granzyme B inside the target was required for proper execution of the death program, I set out to elucidate the mechanism by which granzyme B is transported to its sites of action. Internalization and early trafficking of granzyme B appears to follow a receptor-mediated endocytosis and granzyme B is sequestered inside cytoplasmic vesicles in the target cell until a second signal is given. Evidence obtained from these studies has resulted in a major reassessment of a previously held paradigm regarding the interplay between granzyme B and perforin. Whereas the previous model of granule-mediated apoptosis proposed

that perforin faciltated uptake of granzyme B, I was able to separate the signals provided by these two molecules.

The goals of these studies initially centred on the demonstration of transfer of granzyme B from the CTL to its target. Although intercellular transfer of granule proteins remains unproven, I have made seminal discoveries leading to the proposal of a new model to explain the transport of granzyme B inside the target cell. These discoveries have also led to a re-examination of the mechanism by which perforin exerts its cytolytic activity.

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Doug Scraba and Roger Bradley aided greatly with the electron microscopy. They demonstrated patience to teach me about sample preparation and perfomed the photography. Chris Froelich (Northwest University, Evanston, IL) provided purified human granzyme B and perforin. Prem Seth (NIH) supplied adenovirus. Anti-granzyme antiserum was provided by Dorothy Hudig (University of Nevada, Reno, NV) Microinjection experiments were performed by Kevin Tomaselli (IDUN Pharmaceuticals, La Jolla, CA).

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List of Abbreviations, Nomenclature and Symbols

$\Delta\Psi$	mitochondrial potential gradient
Ad2	Adenovirus Type 2
CCP	cytotoxic cell proteinase
cdMPR	cation-dependent mannose 6-phosphate receptor
ciMPR	cation-independent mannose 6-phosphate receptor
CLSM	confocal laser scanning microscopy
ConA	concanavalin A
CTL	cytotoxic T lymphocyte
DAPI	diamidino-2-phenylindole dihydrochloride
DCI	dicholoisocoumarin
DFP	diisofluoropropyl phosphate
DISC	death inducing signaling complex
DMSO	dimethyl sulfoxide
DPPI	dipeptidylpeptidase I
ECL	enhanced chemiluminescence
EDTA	ethylene diaminetetraacetic acid
endoH	endoglycosidase H
ER	endoplasmic reticulum
Fas	antigen recognized by cytolytic antiserum, Fas
Fas-L	Fas ligand, corresponding ligand for Fas
FITC	fluoroscein isothiocyanate
g	9.81 m/s^2
HPLC	high performance liquid chromatography

H.U.	hemolytic units
M6P	mannose 6-phosphate
MHC	major histocompatibility complex
MPR	mannose 6-phosphate receptor
NK	natural killer (cell)
PAGE	polyacrylamide gel electrophoresis
PARP	poly ADP-ribose polymerase
PBS	phosphate buffered saline
PEL	peritoneal exudate lymphocytes
PFGE	pulsed field gel electrophoresis
pfu	plaque forming units
PT	permeability transition
TBS	Tris-buffered saline
TCR	T-cell receptor
TNF	tumour necrosis factor
TNF-R	tumour necrosis factor receptor
TUNEL	terminal dUTP nick end label

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

CTL MEDIATED CYTOTOXICITY

Cytotoxic T lymphocytes (CTL) are the effectors in cell-mediated immunity that destroy virus infected and neoplastically transformed cells (for reviews see Atkinson and Bleackley, 1995; Berke, 1994, 1995; Kägi et al., 1996). CTL recognize targets in the context of antigen peptides complexed with MHC (major histocompatibility complex) Class I. A close association is facilitated by interaction between the T-cell receptor on the CTL and its accessory molecules (Figure 1.1). After docking of CTL with its target, the apoptotic program is triggered in the target cell and death comes within minutes. There are two types of killing employed by CTL: granule mediated and Fas/TNF-receptor mediated mechanisms. Receptor mediated apoptosis of target cells is signaled through the surface receptor Fas (Apo-1). Engagement of Fas results in stimulation of a protease cascade, or death program, that is apparently innate in all cells. Granule mediated killing utilizes components of the cytolytic granules of CTL to activate the death program. It appears that components of the cytolytic granules utilize the same pathway, but use of the granzymes allows entry into the pathway downstream of surface activation through the Fas receptor.

Directed exocytosis model of granule mediated killing

The cytolytic activity of CTL and NK cells was found to be contained in a lysosome-like granule (Podack and Konigsberg, 1984). In fact, isolated granules from these cells have been shown to be capable of killing target cells in a manner similar to

Figure 1.1 Electron micrograph of cytotoxic T lymphocyte (top) making contact with its target, an EL4 thymoma cell (bottom). A tight association is made by interdigitation of plasma membranes of the two cells forming a shared extracellular space into which the granule components are exocytosed. 18,000x magnification. Electron microscopy performed by Pinkoski and Scraba, unpublished.

death induced by whole CTL. The lytic granule is a specialized vesicle for storage of granule components that undergo regulated secretion once degranulation is induced.

Regulated secretion of granule proteins occurs when an activated CTL engages its target. After conjugation of the two cells, there is a gross intracellular rearrangement of the CTL. Granules of the CTL re-orient toward the interface between killer and target (Zagury et al., 1975; Bykovskaja et al., 1978). The cytolytic granules migrate toward the site of contact and the contents are released into the apposing space. This secretory process forms the basis of the lethal hit model of granule mediated cytotoxicity (Henkart, 1985) in which it was postulated that the molecules contained within the cytoplasmic granules are the lytic effectors that act in concert to bring about target cell apoptosis (Figure 1.2).

Inhibitors of microtubule movement are effective inhibitors of granule exocytosis. It has also been demonstrated that brefeldin A, which inhibits movement of protein from the endoplasmic reticulum to the cis-golgi, does not inhibit primary CTL activity, but does inhibit recycling of CTL for secondary killing (Isaaz et al., 1995). This suggests that the granules of activated T-cells contain functional granule components. Novel protein synthesis is not required for primary killing, but new protein synthesis and transport to the granules is required for "re-loading" of the granules to permit secondary killing activity.

Proteins in the Cytolytic Granule

As mentioned earlier, the cytolytic granule contains all of the machinery necessary to induce target cell death. Granule contents include a family of serine proteinases known as granzymes and the channel forming protein, perforin. There are also a number of regulatory proteins necessary for trafficking to the granule and regulated exocytosis.

Figure 1.2 After conjugation of CTL with its target and exocytosis of granule components, there is a rapid induction of apoptosis in this EL4 target cell. Apoptotic death is characterized by the formation of membrane bound apoptotic bodies (right side of photo) that contain remnants of cytoplasm or nuclei and may include functional organelles. 30,000x magnification. Electron microscopy performed by Pinkoski and Scraba, unpublished.

Perforin

Early observations of cytotoxic cells describe the appearance of ring-like "tubule" pores in the membrane of target cells. (Doumarshky et al., 1980). These structures were determined to be transmembrane channels by electron microscopic analysis (Podack and Dennert, 1983). The authors drew a parallel between the observed NK cell-induced lesions and those lesions formed by some bacterial toxins and proposed the existence of "a general biochemical mechanism" for causing cell death (Podack and Dennert, 1980).

Closer analysis showed that the pore bears structural similarity with C-9, the ninth component of complement (Young et al., 1986a). Complement is a heteropolymeric structure (C1 - C9) that is the mediator of killing in antibody dependent cellular cytotoxicity. More detail on these observations did not come until a number of groups isolated and identified a molecule from the granules of CTL and NK cells (Masson and Tschopp, 1985; Podack et al., 1985; Young et al., 1986b; Liu et al., 1986). These groups identified the pore forming protein perforin as the molecule responsible for forming pores in target cell membranes in a Ca^{2+}-dependent manner. It has long been held that perforin forms channels in the plasma membrane of the target cell through which cytolytic granules proteins pass into the target cell and trigger the cytolytic process (Hameed et al., 1989).

Perforin monomers have a M_r of 65 to 70 kDa, depending on the glycosylation state (Pereschini and Young, 1988) and it was proposed that between 12 and 18 monomers can polymerize to form a transmembrane channel. Given the homology between perforin and complement C-9 (approximately 20% homology involving amino acid residues 101 to 381 of mature human perforin), it was postulated that polyperforin channels may be large enough to form a conduit through which the granule proteinases, or granzymes, gain access to the inside of the target cell.

Surprisingly, the only peptide fragment that was found to contain lytic activity was the N-terminal 34 residues (Ojcius et al., 1991). This region was later refined to the N-terminal 22 residues, which by themselves could cause lysis of red blood cells in a calcium independent manner (Persechini et al., 1992). It is important to note that lytic activity against red blood cells does not correlate to apoptotic activity against nucleated targets.

There were two proposed functions for polyperforin: i) as a macromolecular transport protein that acts at the plasma membrane, forming a pore through which cytolytic effector molecules pass into the target cell and ii) as an effector of endocytic processes. Although the macromolecular pore forming model has received considerable attention, the latter is supported by experimental evidence.

Electron microscopic studies of target cells incubated in the presence of lytic doses of perforin showed that perforin forms pores with an estimated internal diameter of 5-20 nm, large enough for the passage of macromolecular compounds such as granzymes. However, pores of this size have not been observed using sub-lytic concentrations of perforin, leaving open the possibility that the physiologically relevant membrane complex involving perforin may be smaller than the limits of detection for the electron microscope. In fact, Rochel and Cowan (1996) have demonstrated, using 22 residue polypeptides corresponding to the membrane spanning region of perforin, that these domains exhibit self-limiting polymerization properties. Negative cooperativity between membrane domains of perforin limits the number of subunits that may interact to 4 +/- 1, giving rise to an ultimate pore size significantly smaller than that observed by electron microscopic analyses. The pores formed using sub-lytic doses of perforin would provide a channel with an internal diameter of less than 5 nm. A channel of this size would not allow passage of macromolecules. Rather it would only permit the movement of smaller molecules such as ions. Interestingly, one of the first functions proposed for polyperforin was as an ion channel (Podack, 1986). The

possibility remains that perforin may act as membraneolytic agent within the target cell, or that perforin causes a disruption or stimulation of cellular process by its ion channel activity. Perforin ion channel activity may also exist within the target cell.

The importance of perforin in CTL and NK killing has never been questioned but there remained some important questions about the magnitude of the physiological contribution of perforin to the cytolytic process. Red blood cells are very sensitive to perforin-mediated lysis (Henkart, 1984), but nucleated targets require much greater amounts of perforin to be killed (Podack, 1986; Shinkai et al., 1988). It was later discovered that, although perforin alone can cause cytolysis of target cells, it is not able to induce all of the morphological changes observed in a target cell induced to die by CTL or NK cells or by purified whole cytolytic granules (Duke et al., 1989). In the case of the whole granules, the amount of perforin involved in the killing was not, by itself, sufficient to cause lysis. From these data, a model was proposed in which perforin facilitated the transfer of other cytolytic molecules (Hameed et al., 1989).

Granzymes

Pasternack and Eisen (1985), using (^{3}H)DFP (diisofluoropropyl phosphate) binding studies, demonstrated that cytolytic granules from activated T cells contained at least two major serine esterases. A subtractive hybridization approach was used to successfully clone two of these proteinases, CCP1 and 2 (cytotoxic cell proteinase 1 and 2) (Lobe et al., 1985, 1986) using mRNA from activated and resting T cells. CTLA-1 was identified by a similar subtractive hybridization approach using activated T cell cDNA minus B cell (Brunet et al., 1986). CCP1 and CTLA-1 were later determined to be identical. A third granule proteinase was identified by Gershenfeld and Weissman (1986) and named Hannukah Factor based on similarities with Christmas Factor or Factor IX of the blood clotting proteinase cascade.

The following year Masson and Tschopp (1987) reported the identification of nine granule proteinases by N-terminal sequence analysis of purified granule components. In this report the authors also proposed the name "granzyme" for this family of granule serine proteinases (Table 1.1). By this naming scheme, CCP1, or CTLA-1, was renamed granzyme B, CCP2 became known as granzyme C, and Hanukkah Factor was renamed granzyme A. The novel granzymes reported by Masson and Tschopp were named granzyme D, E, F, G, H and K (Table 1.1).

The granzymes are relatively small proteins in the range of 22-35 kDa that are active as monomers, with the exception of granzyme A which has been shown to exist as a homodimer. Granzyme B is the prototypic member of the granzyme family. It maintains a physical characteristic still unique amongst known eukaryotic serine proteases in that it is the only serine proteinase with a preference for Asp residues at the P1 position (the first residue at N-terminal side of the scissile bond). The sequence specificity of granzyme B was predicted based on the molecular model produced by Murphy et al. (1987). The model also predicted a preference for an acidic residue at P3 and a bulky hydrophobic residue at P4. The predictions for cleavage site preference have been confirmed in reports of granzyme B substrates (Table 1.2) (Darmon et al., 1995; Gu et al., 1996 and Chinnaiyan et al., 1996).

Granzyme B is translated as a pre-proprotein with a 22 amino acid residue leader polypeptide that is believed to be removed as granzyme B is exported from the endoplasmic reticulum to the secretory granule. After removal of the leader peptide, cleavage of the N-terminal dipeptide of granzyme B is required to generate an active proteinase. Murphy et al. (1987) used molecular modeling to predict that the Ile-1 of the mature protein makes a critical H-bond with Gly-181 near Ser-183 of the catalytic triad and results in the "opening" of the active site to allow binding and cleavage of substrates. The presence of the Gly-Glu dipeptide at the N-terminus prevents the

GRANZYME	OTHER NAMES AND ACTIVITIES	REFERENCES
granzyme A	Hanukkah Factor	Pasternack et al., 1985 Gershenfeld and Weissman, 1986 Masson et al., 1986
	SE-1	Young et al., 1986b
	CTLA-3	Brunet et al., 1986
	TSP-1	Simon, et al., 1986
	fragmentin-1	Shi et al., 1992
granzyme B	CCP1	Lobe et al., 1986a, 1986b Masson and Tschopp, 1987
	SE2	Young et al., 1986b
	CTLA-1	Brunet et al., 1986
	fragmentin-2	Shi et al., 1992
granzyme C	CCP2	Lobe et al., 1986a, 1986b Jenne et al., 1988a
granzyme D	CCP5	Bleackley et al., 1988 Jenne et al., 1988 Prendergast et al., 1991
granzyme E	CCP3	Bleackley et al., 1988 Jenne et al., 1988b Prendergast et al., 1991
	MCSP-2	Kwon et al., 1988
granzyme F	CCP4	Bleackley et al., 1988 Jenne et al., 1988b Prendergast et al., 1991
	MCSP-3	Kwon et al., 1988
granzyme G	CCP6	Masson and Tschopp, 1987 Jenne et al., 1989
granzyme H	CCPX	Meier et al., 1990 Haddad et al., 1991
granzyme M		
granzyme 3		Hameed et al., 1988

Table 1.1 The granzyme family of serine proteinase found in cytolytic granule of CTL and NK cells.

PROTEASE ACTIVATED BY GRANZYME B	CLEAVAGE SITE	REFERENCES
caspase-3 (CPP32, Yama, apopain)	IETD↓S	Darmon et al., 1995 Martin et al., 1996a Quan et al., 1996
caspase-6 (Mch2)	TEVD↓A	Orth et al., 1996 Srinivasula et al., 1996
caspase-7 (CMH-1, ICE-LAP3)	IQAD↓S	Gu et al., 1996 Fernandes-Alnemri et al., 1996
caspase-8 (MACH, FLICE, Mch5)	VETD↓S	Muzio et al., 1996 Fernandes-Alnemri et al., 1996
caspase-9 (Mch6, ICE-LAP6)	PEPD↓A	Duan et al., 1996b Srinivasula et al., 1996
caspase-10 (Mch4)	IEAD↓A	Fernandes-Alnemri et al., 1996

Table 1.2 Caspase substrates of granzyme B. These proteinases are activated by proteolytic cleavage at the site indicated by an arrow.

interaction between Ile-1 and Gly-181, thus maintaining granzyme B as an inactive zymogen. The contribution of the activation was confirmed when Caputo et al. (1993) first produced recombinant granzyme B only after the DNA encoding the dipeptide was deleted from the open reading frame. In vivo, removal of the activation dipeptide is carried out in the acidic milieu (pH 5.5; Masson and Tschopp, 1985) of the secretory granule by dipeptidyl peptidase I (McGuire et al., 1993; Smyth et al., 1995).

Expression of granzyme B correlates with lytic activity of CTL *in vitro* (Prendergast et al., 1992) and with the activity of infiltrating T lymphocytes in allografts *in vivo* (Mueller et al., 1988). Granzyme B has also been identified as the activity known as fragmentin 2, which was the activity responsible for inducing DNA fragmentation isolated from cytolytic granules of NK cells (Shi et al., 1992a, 1992b).

Other components of the cytolytic granule

In addition to perforin and the granzymes, the cytolytic granule contains a number of other proteins (Table 1.3). Calreticulin is upregulated in CTL upon cellular activation (Burns et al., 1992) and has been co-localized with granzymes in cytolytic granules by immunocytochemistry (Pinkoski and Bleackley, unpublished observations). The presence of calreticulin in the secretory granule of CTL was discovered by its co-purification with perforin (Dupuis et al., 1993). Calreticulin is a Ca^{2+} binding protein that has been postulated to provide a Ca^{2+} sink to prevent premature polymerization of perforin within the granule and may also provide a ready source of Ca^{2+} at the time of degranulation (see Michalak et al., 1992; Bleackley et al., 1995). A C-terminal KDEL sequence acts as an endoplasmic retention signal to return calreticulin back to the ER after shuttling to the *cis*-golgi. How calreticulin overcomes retention in the ER to be transported to the cytolytic granule is not yet known.

PROTEIN	ACTIVITY	REFERENCE
perforin (cytolysin)	pore-forming protein	Podack et al. 1985 Young et al. 1986a Groscurth et al. 1987
granzymes	proteinases	see Table 1.2
mannose 6-phosphate receptor (MPR)	protein targeting	Burkhardt et al. 1990 Peters et al. 1991 Griffiths and Isaaz 1993
chondroitin sulfate	complexes with granule proteins	Tschopp and Masson 1987 Masson et al. 1990 Peters et al. 1991
calreticulin	perforin and Ca^{2+} binding protein	Burns et al. 1992; Dupuis et al. 1993
TIA-1	RNA binding protein	Anderson et al. 1990; Tian et al. 1991
leukalexin	TNF-like cytokine	Liu et al. 1987
leukophysin	granule mobility	Abdelhaleem et al. 1991, 1996
cathepsin D	lysosomal protease	Tschopp and Nabholz 1990
cathepsin C	dipeptidyl peptidase I	McGuire et al., 1993
arylsulfatase	lysosomal enzyme	Tschopp and Nabholz 1990
β-glucoronidase	lysosomal enzyme	Tschopp and Nabholz 1990
ATPase H^+ antiporter	acidification	Kataoka et al. 1994
lamp-1	lysosomal protein	Peters et al. 1991
lamp-2	lysosomal protein	Peters et al. 1991
CD63	lysosomal protein	Peters et al. 1991

Table 1.3 Constituents of the cytolytic granule of CTL and NK cells. Granules are believed to be dual purpose vesicles that are similar to lysosomes and are active components of regulated secretion (Burkhardt et al., 1990).

Chondroitin sulfate is believed to provide a protection from self-lysis due to perforin polymerization in the killer cell. In the acidic state of the granule, chondroitin sulfate binds to perforin. Once degranulation occurs the granule contents are exposed to the neutral pH of the extracellular milieu. At the neutral pH chondroitin sulfate and perforin dissociate and perforin is available for homopolymerization. Chondroitin sulfate-granzyme complexes may also represent a means to render the granzymes reversibly inactive while inside the killer cell.

Mannose 6-phosphate receptor (MPR) mediates the trafficking of the high mannose containing proteins to lysosomes. In CTL and NK cells, MPR directs trafficking of the high mannose containing granzymes to the secretory granule (Peters et al., 1991). The ability to differentiate between lysosomes and secretory granules in CTL and NK cells is not known, but may be due to selective contribution from each of the cation-dependent and -independent MPR (Hill-Rehfeld, 1995).

Dipeptidyl peptidase I (DPPI), also known as cathepsin C, is a cysteine proteinase that specifically removes N-terminal dipeptides. DPPI is a modifying protease that is responsible for proteolytic activation of granzyme B (McGuire et al., 1993; Smyth et al., 1995) and granzyme A (Kummer et al., 1995).

Leukophysin is involved in granule motility, which is required for re-orientation after conjugation of killers with targets in preparation for degranulation (Abdelhaleem et al., 1991, 1996). TIA-1 is a 15 kDa protein specific to cytolytic granules of CTL and NK cells that has RNA binding capability but unknown function in cytolysis (Tian et al., 1991). Other granule proteins include lysosomal enzymes cathepsin D, β-glucuronidase and arylsulfatase as well as the lysosomal proteins lamp-1, lamp-2 and CD63 (Peters et al., 1991).

Mechanism of granzyme mediated cytolysis

The focus of the studies presented herein is on apoptosis induced in target cells by cytotoxic T lymphocytes (CTL). Induction of the cell death program is a strategy utilized by CTL and natural killer (NK) cells as a means to destroy virus infected or tumour cells. Cytolysis of targets during cell mediated cytotoxicity is due to the transfer of a number of cytolytic effector molecules from a CTL or NK cell into a target. Since the introduction of trypsin, chymotrypsin or proteinase K into a target cell resulted in cytolysis, it was believed that death was a result of proteolytic degradation (Williams and Henkart, 1994). A similar mechanism was proposed for granzymes in target cells. However, with the identification of caspase activation as a critical step in granzyme-mediated apoptosis, a new model is emerging that is much more complex and is tightly regulated by the target cell. Rather than merely degradative, cleavage of target cell substrates by CTL proteases results in the activation of protease cascades within the target. The contribution of the target in its own demise was once overlooked, but is now recognized as vital for proper execution of the death program.

In support of the notion that the granzymes induce apoptosis, in part, by cleaving cytosolic proteins, granzyme B has been reported to activate the CPP32-like members of the caspase family (Table 1.4). For example, caspase-3 (CPP32) is processed in murine targets treated with CTL (Darmon et al., 1995) and targets exposed to granzyme B and perforin contain processed caspase-1, -3 and -7 (Shi et al., 1996; Chinnaiyan et al., 1996). Thus, it is implied that granzyme B gains access to the interior of the target cell where it can cleave and activate these substrates. Activation of the caspases has been shown to be essential in DNA fragmentation (Darmon et al., 1996) and more recently for externalization of phosphatidylserine in the plasma membrane of cells undergoing apoptosis (Martin et al., 1996).

Activated CPP32 cleaves a number of DNA repair enzymes, including poly ADP-(ribose)polymerase (PARP; Nicholson et al., 1995; Tewari et al., 1995) and

CASPASE	FORMER NAME(S)	ACTIVATED BY:	SUBSTRATE(S)
caspase-1	ICE		pro-IL1β pro-caspase-3 pro-caspase-4
caspase-2	ICH-1		
caspase-3	CPP32/apopain Yama	granzyme B caspase-1	PARP DFF DNA-PK SRE/BP rho-GDI
caspase-4	ICErel-II TX ICH-2	caspase-1	
caspase-5	ICErel-III; TY		
caspase-6	Mch-2	caspase-7 granzyme B	lamin A
caspase-7	Mch-3 ICE-LAP3 CMH-1	granzyme B	PARP pro-caspase-6
caspase-8	FLICE MACH Mch5	granzyme B	
caspase-9	ICE-LAP6	granzyme B	PARP
caspase-10	Mch4	granzyme B	

Table 1.4 Proteins of the ICE/CED-3 family have been renamed caspases and a number designation (Alnemri et al., 1996). For references see review by Nagata, 1997

DNA dependent protein kinase (Song et al., 1996a, 1996b). In addition to the cytoplasmic substrates of granzyme B there is an increasing body of evidence suggesting that granzyme B may act in the nucleus. In two independent studies, granzyme B was shown to bind to nuclear proteins (Pinkoski et al., 1996; Trapani et al., 1996). At least one of these potential substrates is associated with heterochromatin and the perinucleolar region of the nucleus (Pinkoski et al., 1996).

Fas/TNF-Receptor mediated killing

The granule exocytosis model of cell mediated cytotoxicity is sufficient to explain many instances of induced death of target cells, but it cannot explain the observation that some peritoneal exudate lymphocytes (PEL) are able to kill efficiently without detectable levels of perforin or granule components (Helgason et al., 1992). Granule-independent cytotoxicity would later be explained by the identification of the Fas surface antigen (see Nagata and Golstein, 1995; Nagata, 1997).

The Fas surface receptor, also known as Apo-1, was identified as the antigen recognized by cytolytic antisera (Trauth et al., 1989; Yonehara et al., 1989). Addition of these antisera into the media of sensitive cells resulted in rapid induction of apoptosis. The corresponding antigen, Fas, was found to be a novel member of the tumour necrosis receptor superfamily (Oehma et al., 1992; Suda et al., 1993). Transfection of the cDNA encoding the Fas antigen conferred sensitivity to apoptosis induced by anti-Fas antiserum (Oehm et al., 1992). Identification of the corresponding Fas ligand (Fas-L) showed it to be a novel member of the tumour necrosis superfamily that possesses cytolytic activity toward cell expressing surface Fas (Suda et al., 1993).

The death-inducing signaling complex (DISC)

Engagement of Fas by Fas-L results in trimerization of Fas and recruitment of a number of accessory molecules to form the death-inducing signaling complex, DISC

(Kischkel et al., 1995). Signaling through the DISC results in the activation of a number of proteases, primarily members of the caspase family (Schlegel et al., 1995; Chow et al., 1995). Activation of caspases ultimately drives the apoptotic death program.

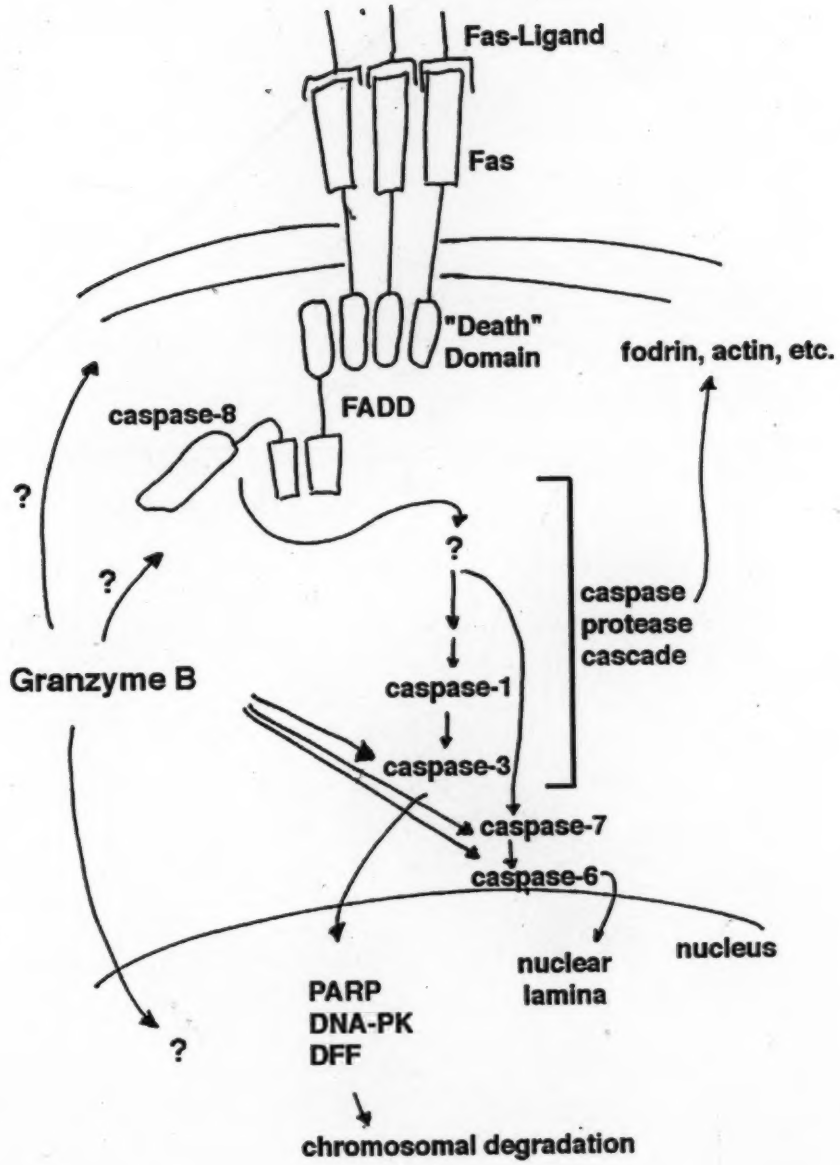
Within seconds of stimulating trimerization of Fas there is an alphabet soup of acronyms recruited to the DISC (Figure 1.3). Four major cytotoxicity-dependent Apo-1-associated proteins (CAPs) were detected by two dimensional electrophoresis (Kischkel et al., 1995). CAP 1 and 2 were identified as the death related proteins MORT1/FADD (Fas-associated death domain) (Chinnaiyan et al., 1995). The death domain of Fas had earlier been identified as a conserved region that is required for inducing apoptosis (Itoh and Nagata, 1993). Other components of the DISC include proteins that associate with the Fas molecule or the analogous signaling complex involving the TNF receptor (see review by Nagata, 1997).

Identification of FLICE/MACH (caspase-8) as a component of the DISC is an important discovery in our understanding Fas-signaling, because it is the first demonstration of a caspase that is directly involved with the surface receptor complex (Boldin et al., 1996; Muzio et al., 1996). Caspase-8 activates downstream caspases such as caspase-3. Therefore, caspase-8 may be a direct link between ligation of the Fas surface receptor, recruitment to the DISC and subsequent activation of downstream caspases.

PROGRAMMED CELL DEATH AND APOPTOSIS

Programmed cell death, or apoptosis, is now known to occur in a diverse array of physiologically important systems. Apoptosis may be the type of death that occurs in as much as 95% of all cell death occurring in vertebrates (Jacobson et al, 1997). From a physiological standpoint, it is advantageous for an organism to remove unwanted cells by apoptosis rather than necrosis, since apoptotic death does not elicit

Figure 1.3 Schematic illustration of the death program. It appears that a caspase proteinase cascade is fundamental component of the apoptotic machinery. The tumour necrosis factor receptor (TNF-R) works through a system analogous to Fas. Signaling through Fas and TNF-R is facilitated by accessory molecules that make up the DISC, or death inducing signaling complex. Proposed sites of granzyme B action are indicated by question marks.


Fas-Ligand
Fas
"Death"
Domain
FADD
caspase-8
fodrin, actin, etc.
?
?
?
caspase
protease
cascade
Granzyme B
caspase-1
caspase-3
caspase-7
caspase-6
nucleus
nuclear
lamina
PARP
DNA-PK
DFF
?
chromosomal degradation

an inflammatory response. This is due to the rapid engulfment of apoptotic cells prior to cell lysis. In contrast, cytolysis by necrosis results in the spillage of intracellular proteins into the extracellular milieu and the possibility of mounting an inflammatory response at the site of injury or an immune response against antigens that are normally sequestered from immune surveillance.

Necrosis is often a result of severe cellular insult and is characterized by loss of membrane integrity and upset of osmotic control, which causes a swelling of the cell and ultimately leads to the osmotic lysis. Death by apoptosis, on the other hand, requires active participation by the dying cell. Once induced to die, the apoptotic cell maintains membrane integrity, but exhibits blebs that form into apoptotic bodies. These bodies are membrane bound buds that are pinched off of the dying cell to be engulfed and digested by neighbouring cells.

Apoptosis was first described morphologically in adrenal cortex tissue sections in which cell death was induced by withdrawal of adrenocorticotrophic hormone, ACTH (Kerr et al., 1972). The distinction noted in this type of death is that the breakdown products of the dying cells are contained in membrane bound bodies until the apoptotic bodies are engulfed by neighbouring cells. A further distinction was noted in 1980, when Wyllie reported that an endogenous nuclease activity was responsible for apoptotic death of glucocorticoid stimulated thymocytes (Wyllie, 1980). Wyllie suggested that "cell deletion seems to be 'programmed'" and required active cellular processes of the dying cell including endogenous endonuclease activity (Wyllie, 1980) and novel protein synthesis (Wyllie et al., 1984).

It is now recognized that apoptosis is the means by which the majority of cells die in our bodies. During development embryonic re-modeling such as the removal of interdigitating cells to form fingers and toes occurs by apoptosis (Jacobson et al., 1997). Control of cell death by an innate "death program" is seen in many cells ranging from invertebrates to eukaryotic zygotes (Steller, 1995; Weil et al., 1996; Shaham and

Horvitz, 1996). In vertebrates the selection and deletion of cells in the immune system is regulated at virtually every level by apoptotic death. Generation and maintenance of a high fidelity immune repertoire requires removal of self-reactive B- and T-cells through negative selection as well as removal of non-reactive cells that do not qualify for positive selection. Clearance of infected or aberrant cells by cell mediated cytotoxicity is effected by triggering the target cell's innate death program. Even removal of lymphocytes by clonal deletion following expiration of their usefulness occurs by apoptosis.

The B-cell lymphoma-2 (bcl-2) gene product

Follicular B-cell lymphoma in humans is frequently associated with the translocation t(14;18). The translocated gene was cloned, identified and designated *bcl-2*, for B-cell lymphoma-2 (Tsujimoto et al., 1984; Cleary et al., 1986). This shifts the *bcl-2* gene from its locus at 18q21 to 14q32, juxtaposed with the Ig heavy chain gene, resulting in deregulated overexpression of the translocated bcl-2 gene in pro- and pre-B cells.

Overexpression of bcl-2 in transgenic mice results in enhanced survival of thymocytes from these animals after apoptotic stimuli such as α–CD3, glucocorticoid and growth factor deprivation (McDonnell et al., 1989; Nuñez et al., 1990; Strasser et al., 1991, 1994; Siegel et al., 1992). Proliferation of these cells appears to be unaffected. Cells undergoing negative selection appear to have increased resistance to apoptotic stimuli, but self-reactive T-cells are not observed in the periphery. In similar experiments, bcl-2 overexpression also appears to protect from deletion of those B-cells that fail to acquire a productively rearranged surface Ig and results in accumulation of B-cells and formation of splenic nodules (McDonnell et al., 1989).

Homozygous null mutant bcl-2 (bcl-2-/-) mice display T-cells with increased susceptibility to apoptotic stimuli, renal failure due to polycystic kidneys and

hypopigmentation in hair follicles (Veis et al., 1993). The disturbance of pigment production indicates that the bcl-2-/- genotype results in a defective redox-regulated melanin production system and supports the earlier finding that bcl-2, localized to the inner leaflet of the mitochondrial membrane (Hockenbery et al., 1990), can act after the generation of superoxide and peroxides to prevent resultant lipid peroxidation and DNA degradation (Hockenbery et al., 1993). Bcl-2 has also been shown to prevent cell death resulting from hypoxic shock (Jacobson and Raff, 1995; Shimizu et al., 1995).

The discovery of *bcl-2* represents a pivotal point in our understanding of apoptosis. This protein appears to be responsible for protection from many death stimuli (Hockenbery et al., 1990; Bissonette et al., 1992; Cuende et al., 1993; Chiou et al., 1994). The bcl-2 protein is well conserved throughout evolution. Human bcl-2 protein can functionally substitute for mutants of the ced9 gene, the *C. elegans* bcl-2 homologue, by inhibiting the cell death program (Hengartner et al., 1992; Vaux et al., 1992; Hengartner et al., 1994). Experiments have shown that bcl-2 acts at points both upstream and downstream of caspases and appears to be one of the key regulatory proteins in the induction of, and protection from, apoptosis (Nuñez and Clarke, 1994; Reed, 1994; Cory, 1995).

BIOCHEMICAL FEATURES OF APOPTOSIS

Salient features of the molecular mechanisms of cell death are now becoming clear. Although the upstream stimuli are widely varied, the end result involves the systematic dismantling of the dying cell. This involves many morphological and biochemical hallmarks, including loss of membrane asymmetry, cytosolic acidification, disintegration of the cytoskeleton and nuclear lamina and DNA fragmentation. The molecular ordering of the events in apoptosis remains contentious, but it appears that mitochondrial changes result in the activation of cellular proteases. These early events precede loss of membrane asymmetry and breakdown of the nucleus and the

cytoskeleton, all of which precede loss of membrane integrity (Gottlieb et al., 1996; Meisenholder et al., 1996).

Mitochondrial events

Involvement of the mitochondria in apoptosis was first proposed when the anti-apoptotic protein bcl-2 was localized to the inner membrane leaflet of the respiratory organelle (Hockenbery et al., 1990). The notion that the mitochondria may provide a control centre for many of the apoptotic processes was suggested when Newmeyer et al. (1994), using a cell free system of apoptotic death of *Xenopus* oocytes, showed that apoptosis required mitochondrial fractions. Interestingly, the positive apoptotic effects of the mitochondrial fractions are inhibitable by bcl-2.

Active mitochondria possess a transmembrane potential ($\Delta\Psi$) that is disrupted during apoptosis (Kroemer et al., 1997). Loss of $\Delta\Psi$ is caused by an opening of the pores that regulate the permeability transition (PT) (Zamzami et al., 1995a; Marchetti et al., 1996a). Inhibition of the PT with bongkrekic acid inhibited apoptosis and stimulating the PT with protoporphyrin IX induced apoptosis (Zamzani et al., 1996a; Marchetti et al., 1996a; 1996b). Reduction of the PT and subsequent loss of $\Delta\Psi$ represent an irreversible step in the commitment to apoptosis that results in the generation of reactive oxygen species (Zamzami et al., 1995b) and the release of an apoptotic protease, AIF (apoptosis-inducing factor), which has characteristics of the caspases. The release of AIF is inhibitable by overexpression of *bcl-2* (Susin et al., 1996) and is believed to activate caspase-3, which in turn induces nuclear changes including DNA fragmentation (Zamzami et al., 1996b).

Cytochrome c is a normal constituent of mitochondria. Liu et al. (1996) found that cytochrome c is necessary for the induction of apoptosis in the cell free system in which they added purified cytoplasmic fraction to isolated nuclei and assayed DNA

fragmentation. They found a potent apoptosis inducing factor that corresponded to cytochrome c. Conversely, cytoplasmic fractions immunodepleted of cytochrome c no longer contained the apoptosis inducing activity. Evidence from two recent studies suggest that *bcl-2* may prevent the release of cytochrome c from the mitochondria (Yang et al., 1997; Kluck et al., 1997).

Proteases in Apoptosis

As the biochemical machinery responsible for carrying out the death program varies throughout the diversity of cell types and apoptotic stimuli, one of the most unifying features is the necessity for proteases (see Martin and Green, 1995a; Nagata, 1997). The contribution of proteases toward cell death was originally believed to be as a degradative activity, but it is now clear that apoptotic proteases are also involved in the activation of apoptotic substrates, including other proteases and nucleases.

One of the most useful biological systems in which to study programmed cell death is the nematode *Caenorhabditis elegans*. The immature worm consists of 1090 cells, of which exactly 131 are programmed to die, resulting in a mature worm with 959 cells. *C. elegans* has proved to be a valuable system from which much of our understanding of apoptosis in higher organisms has been expanded. In the late 1980's fourteen genes, termed *ced* for *C. elegans* death, were identified from *C. elegans* that were involved in some capacity in cell death in the developing worm (reviewed in Ellis et al., 1991). The first of the *ced* genes implicated in apoptosis was *ced* 9, that was identified by mutational analyses (Hengartner et al., 1992). *ced* 9 protected cells from apoptosis by regulating *ced* 3 and *ced* 4. Loss of function mutants of *ced* 9 underwent inappropriate cell death which resulted in embryonic lethality. *ced* 9 was later found to be a functional homologue of the mammalian anti-apoptotic protein, bcl-2 (Hengartner and Horvitz, 1994).

The first pro-apoptotic of the *ced* genes identified was *ced* 3. Overexpression of *ced* 3 resulted in inappropriate apoptosis. *ced* 3 is the nematode homologue of the mammalian interleukin 1β-converting enzyme (ICE) (Yuan et al., 1993). ICE and *ced* 3 are also functional homologues as overexpression of either can induce cell death in Rat-1 fibroblasts (Miura et al., 1993). Since the discovery of the involvement of *ced* 3 and ICE in cell death there have been a number of additional members of this family of cysteine proteases identified. The ICE family of proteases has recently been renamed caspases (see Table 1.4) for cysteine, aspartic acid cleaving proteases due to the preference for aspartic acid at the P1 position of the scissile bond (Alnemri et al., 1996).

All known members of the caspase family are expressed as preproproteins that require proteolytic processing, after which they may direct a second proteolytic event in an autocatalytic manner. In healthy cells the caspases remain in an inactive form and these two processing steps are required to generate the active, heterotetrameric proteinase. After processing, caspases are found in an $\alpha_2\beta_2$ tetrameric form with subunits of 17 and 11 kDa (Walker et al., 1994).

Although induction of apoptosis by overexpression of caspase-1 in Rat-1 fibroblasts was the first implication of a caspase as a mediator of apoptosis (Miura et al., 1993), the field exploded with the discovery of CPP32, or apopain, now known as caspase-3 (Schlegel et al., 1996). Cleavage of the DNA repair enzyme poly ADP-ribose polymerase by caspase-3 was a major discovery in this field since at this time there had been no downstream effects or activities attributed to any of the caspases. However, since this first demonstration of caspase-3 activation as a necessary step in the biochemical pathway leading to cell death, the flood gates have opened on an extremely rapidly moving and exciting stream of research.

Shortly after its discovery, caspase-3 was shown to be cleaved by granzyme B *in vitro* and more recently *in vivo* in apoptotic targets of CTL (Darmon et al., 1995; Chinnaiyan et al., 1996). The involvement of caspase 3 in apoptosis was originally characterized by the cleavage of the DNA repair enzyme poly ADP-ribose polymerase (PARP) (Nicholson et al., 1995; Tewari et al., 1995), but the cleavage of PARP by caspase-3 could not, by itself, explain the magnitude of the DNA fragmentation observed in apoptotic cells. An additional nuclear activity of caspase-3 has recently been described that may better explain the role of caspase-3 in DNA fragmentation during apoptosis. Liu et al. (1997) have identified a caspase-3 activated protein that stimulates an endonuclease that appears to be responsible for whole scale DNA fragmentation. The presence of a caspase-3-activated endonucleolytic activity explains the observation that activation of caspase-3 results in profound DNA fragmentation in apoptotic cells and also that inhibition of caspase-3 itself can entirely abrogate this fragmentation.

The caspase protease cascade is now recognized as a major component of the apoptotic program. Caspase-8 associates with the DISC and activates downstream caspases which, in addition to cleavage of PARP and DFF by caspase-3, are responsible for degradation of the nuclear lamina and the cytoskeleton (see Martin et al., 1995b; Nagata, 1997). Proteolytic degradation of the nuclear lamina is a key event in the dismantling of nucleus and results in the nuclear condensation that is a morphologic characteristic of apoptotic cells. Caspase-dependent cleavage of fodrin results in breakdown of the cytoskeletal framework of the cell.

Loss of membrane asymmetry

In a healthy cell there is little or no phosphatidylserine present on the outer surface of the plasma membrane (see Op den Kamp, 1979; Swairjo and Seaton, 1994). Membrane asymmetry is maintained by a phospholipid translocase, also termed a

"flippase," activity that returns PS to the inner face of the membrane. Display of PS on the outside of a cell is an early event in apoptosis and is a signal to neighbouring cells that the cell is undergoing apoptosis. This is a critical step in physiological systems because dying cells are engulfed while apoptotic bodies are still membrane bound (Kerr et al., 1972). In tissue culture systems where there are no phagocytes, dying cells undergo secondary necrosis, which is typified by loss of membrane integrity and lysis of the dying cell. PS on the dying cell is recognized by CD36 and thrombospondin on the surface of macrophages (Savill et al., 1992). Transfection of CD36 gene into non-professional phagocytes, melanoma and fibroblast cells, was sufficient to confer a professional phagocytic phenotype (Ren et al., 1995). Recognition of a dying cell is believed to be mediated through the membrane bound CD36 of the macrophage which interacts with thrombospondin, and in turn interacts with vitronectin receptor and PS (Savill et al., 1990, 1992). The display of PS on the outside of a cell was an important discovery because it allows for detection of an early event in apoptosis through the use of the PS-binding protein annexin V (Martin et al., 1995b; see also Swairjo and Seaton, 1994).

Acidification of the cytosol

Using CTLL-2, an IL-2 dependent CTL line as a model system, Li and Eastman (1995) showed that these cells, while undergoing apoptosis, display a consistent drop in intracellular pH. Acidification of the cytosol was found to be a result of an alteration in the vacuolar ATPase Na^+/H^+ antiport molecule. The "set-point" of the Na^+/H^+ antiporter was fully functional, but was set 0.7 pH units lower than normal physiological levels (6.3-6.5 compared to 7.2). These researchers also observed that acidification through Na^+/H^+ antiport control was not necessary for apoptosis, but appeared to serve a regulatory role in the progression of apoptotic processes.

Gottlieb et al. (1995, 1996) described an acid (pH < 6.8) dependent endonuclease and proposed that early acidification may facilitate genome digestion. The endonuclease is independent of Ca^{2+} and Mg^{2+} which is consistent with an acid-dependent nuclease activity described by Li and Eastman (1995).

In an attempt to determine the order of some of the events in apoptosis, Meisenholder et al. (1996) used Fas ligation to induce apoptosis in Jurkat T lymphoblast cells. These researchers observed that acidification occurred concurrently with loss of membrane asymmetry, which places both events early in stages of cell death. It was also shown that the overexpression of the anti-apoptotic protein bcl-2 was able to prevent acidification and as well as other aspects of cell death. Since all of the anti-apoptotic effects of bcl-2 are not known, it would be premature to conclude from these studies that, contrary to the studies of Li and Eastman (1995), acidification leads to further downstream events in cell death. It is clear, however, that bcl-2 exerts some effect on intracellular acidification.

Cytoskeletal breakdown

Membrane blebbing and formation of apoptotic bodies are classic morphological features of apoptotic death (Kerr et al. 1972). Despite profound changes in the shape and size of a dying cell, the plasma membrane is maintained intact. An underlying mechanism to explain these observations was put forth by Martin et al. (1995b) who demonstrated that non-erythroid spectrin, or fodrin, was cleaved during apoptosis. The T cell hybridoma line A1.1 undergoes apoptosis in response to a number of physiologically relevant stimuli, including the corticosteroid dexamethasone, engagement of the T cell receptor complex via α-CD3, and staurosporine. Regardless of the death stimulus, there was a specific cleavage of the 240 kDa α subunit of fodrin into signature fragments of 150 and 120 kDa.

Fodrin is a 240 kDa protein that interacts with actin (Harris et al., 1990), calmodulin (Harris et al., 1988) and CD45 (Iida et al., 1994) and provides a structural framework for the cytoskeleton. Cleavage of fodrin by the Ca^{2+}-dependent protease, calpain may play a role in cytoskeletal rearrangement during such events as receptor activation and apoptosis (Harris et al., 1990; Stabach et al., 1997). With the cleavage of fodrin the cytoskeletal framework is disassembled, resulting in cell shrinkage and the formation of apoptotic bodies following membrane blebbing.

The apoptotic cleavage site of fodrin, which differs from the calpain cleavage site, has been proposed with Asp at the P1 position (Nath et al. 1996). The identified cleavage site, DETD↓SK, corresponds to caspase recognition sites. Cleavage of fodrin at this site is inhibitable by wide spectrum caspase inhibitors in growth factor withdrawal-induced apoptosis of neuronal PC12 cells (Nath et al., 1996).

Loss of cell volume

Breakdown of the cytoskeleton is accompanied by a concomitant loss in cell volume. While cleavage of fodrin can explain a loss of cytoskeletal integrity, it cannot fully explain cytoplasmic shrinkage. Cidlowski et al. have recently shown that efflux of K^+ ions is responsible, in whole or in part, for cell shrinkage (J.A. Cidlowski, NIH, personal communication). Loss of one K^+ ion results in the loss of 300 H_2O molecules, resulting in a drastic reduction in cell volume. By performing apoptotic assays in isotonic K^+ (approximately 140 mM) loss of cell volume was inhibited. This also affected other hallmarks of apoptosis including activation of the caspases. Shrinkage of cells undergoing apoptosis may also facilitate engulfment and subsequent disposal by neighbouring cells.

Nuclear events in target cell apoptosis

Nuclear disintegration is an essential step in the destruction of a target cell. This is evident since perforin can "kill" anuclear red blood cells (Henkart, 1985); but other granule components are necessary to bring about nuclear changes in the target cell observed in vivo (Shinkai et al., 1988; Shi et al., 1992). This includes cleavage of DNA into the hallmark nucleosomal ladder sized fragments as well as proteolytic degradation of the nuclear lamina.

The studies showing that the disintegration of the nucleus is a dispensable event in apoptosis (Ucker et al., 1992; Jacobson et al., 1994; Nakigawa et al., 1995) is an interesting observation, but suggests that rather than being dispensable, nuclear events may lie downstream in the apoptotic process and may not be required for other biochemical hallmarks of apoptosis. Since most eukaryotic cells possess intact nuclei and a full complement of DNA, it is likely that *in vivo,* nuclear disintegration and DNA fragmentation are physiologically significant and perhaps nuclear disintegration may represent one of the final steps in the death of a cell.

DNA fragmentation

Perhaps the most heralded feature of apoptosis is the internucleosomal "laddering" pattern of DNA fragmentation (for review see Sellins and Cohen, 1995). This phenomenon was described by Wyllie (1980) in glucocorticoid treated thymocytes and has become a valuable means to differentiate between apoptosis and necrosis. DNA damage in targets of CTL is induced within minutes of contact (Russell and Dobos, 1980) as a result of activation of an intracellular endonuclease in the target (Duke et al., 1983). In hormone induced apoptosis, the onset of DNA fragmentation is somewhat slower than that induced by CTL and there is a requirement for novel macromolecular synthesis (Wyllie et al., 1984).

In 1993, Oberhammer et al. demonstrated that genome digestion leading to the signature 180 bp multimers proceeds through an intermediate cleavage step. On a number of fibroblast cell lines induced to die by various means, including serum starvation and etoposide, these researchers used field inversion gel electrophoresis to demonstrate that the DNA is cleaved into 50 to 300 kbp fragments prior to internucleosomal fragmentation.

The most common requirement for the apoptotic endonuclease activity is a dependence on Ca^{2+} and Mg^{2+} and the activity is inhibitable by Zn^{2+} (Wyllie, 1980; Cohen and Duke, 1984; Arends, 1990; Khodarev and Ashwell, 1996). DNase I has been implicated in apoptosis and DNase II has been shown to be active in acidic conditions similar to those observed in apoptotic nuclei. Cyclophilin has been shown to possess Ca^{2+}-dependent nuclease activity in the steroid treated thymocyte system (Montague et al., 1995), but the physiological relevance of this activity is not known.

Perhaps the most compelling evidence toward the identification of an apoptotic endonuclease activity is the isolation of DNA fragmentation factor, DFF (Liu et al., 1997). DFF, after activation by caspase-3, induces nuclear DNA fragmentation by subsequent activation of a downstream endonuclease. This is the first report of a nucleolytic activity, direct or indirect, that lies within the known apoptotic pathways.

Nuclear condensation

Along with fragmentation of apoptotic cell DNA, Wyllie and colleagues observed a margination of the chromatin and subsequent condensation of the nucleus (Kerr et al., 1972; Wyllie and Morris, 1982; Wyllie et al., 1984). Nuclear condensation is believed to be the result of breakdown of the nuclear lamina (Lazebnik et al., 1993; Oberhammer et al., 1994). Lamins A, B and C are the principal components of the lamina that makes up the nuclear envelope.

Cleavage of lamin A and C, two splice variants of the same gene, is carried out by caspase-6 (Lazebnik et al., 1995; Orth et al., 1996; Takahashi et al., 1996). Caspase-3, another caspase with known nuclear activity, is not directly responsible for cleavage of lamin A (Takahashi et al., 1996), but has been shown to activate caspase-6. Cleavage of lamins A, C and B precede DNA fragmentation and it is believed that disintegration of the nuclear lamina facilitates further dismantling of the nuclear components, including fragmentation of DNA (Neamati et al., 1995; Rao et al., 1996).

OBJECTIVES OF THESE STUDIES

My primary objective was to demonstrate the transfer of molecules, namely granzyme B from the CTL to its target. This has long been somewhat of a holy grail for scientists studying cell mediated cytotoxicity. However, to reach this goal I first set out to determine if granzyme, given access to the target cell, could recognize and bind to intracellular proteins. This approach, if successful would give us confidence that granzyme B did, in fact, enter the target cell and act upon intracellular substrates and it would also provide us valuable information regarding the intracellular localization of potential substrates. Knowing where in the cell substrates reside would facilitate their isolation and identification.

As these studies progressed and substrates for granzyme B were being identified, a new and exciting possibility presented itself. Perforin had long been thought to form macromolecular channels in the target cell through which the granzymes passed and gained access to the cytoplasm of the target. However, this phenomenon had never been supported by experimental evidence. It was suggested that perhaps granzyme B gained access to the target cell independently of perforin. We addressed this issue using an *in vitro* model system in which purified granzyme B is added directly to the target cell media. We then used confocal laser scanning microscopy to follow immunocytochemically labeled granzyme B in the target. Using

this powerful technology, we determined the cellular locale of granzyme B to be inside the target cell in a cytoplasmic vesicle.

The route of granzyme B transport in the target cell remained to be elucidated. This problem was broken down into two sections: entry of granzyme B and subsequent trafficking of granzyme B leading to death of the target. To study the entry of granzyme B we employed some classical techniques utilized to determine the presence of specific protein receptors versus the non-specific entry by fluid phase engulfment. Utilizing temperature-dependent inhibition of uptake and pH-dependent dissociation of receptor bound molecules we were able to determine that entry of granzyme B is likely mediated by interaction with a specific receptor.

The study of granzyme B uptake into the target was very successful and we were faced with a new series of questions. Granzyme B could gain access to the target cell, but was not sufficient to induce apoptosis. Given that perforin was not needed for granzyme entry but was required to induce death in the presence of granzyme B, how is perforin acting in the cell? The study of a physiologically relevant role for perforin was facilitated by the discovery that a replication deficient strain of adenovirus (Ad2) could replace perforin and induce apoptosis in granzyme B treated targets.

The studies presented in this thesis are focused on the events concerning entry of trafficking of granzyme B in the target cell. How does granzyme B get into the target cell and where does it go once inside? What triggers the release of granzyme B from its cytoplasmic compartment and how does the granzyme get into the nucleus? How is granzyme B brought into contact with substrates? Although the primary goal of demonstrating transfer of granzyme B from CTL to target was never obtained, we have uncovered a novel system of entry of granzyme B and developed a model in which granzyme B is subjected to trafficking within the target cell. In doing so, the target cell is exerting yet one more level of control in cell mediated cytotoxicity.

CHAPTER II

EXPERIMENTAL PROCEDURES

CHAPTER II

EXPERIMENTAL PROCEDURES

Cell lines and animals

The cytotoxic T lymphocytic cell lines MTL2.8.2, and the target cell lines EL4 (murine thymoma), Jurkat (human lymphoma), L1210 (murine leukemia), YAC-1 (murine leukemia) and P815 (murine mastocytoma) were grown in RPMI 1640 with 5% fetal calf serum, 2-mercaptoethanol (10mM), penicillin 100 U/ml), streptomycin (150µg/ml) in 10mM Hepes pH 7.2. COS M5 cells were cultured in Dulbeco's Modified Eagle's Medium containing 10% fetal calf serum, penicillin 50U/ml), streptomycin (50µg/ml). All cells were incubated at 37°C in 5% CO_2 unless otherwise stated.

Protein purification

Whole cell protein lysates were isolated by either low salt buffer (10 mM NaCl, 15 mM KCl and 10 mM Tris HCl pH 7.4) and physical disruption by Dounce homogenization or by direct lysis in low salt buffer containing Triton X-100 (0.5%). Nuclear pellets were removed by low speed centrifugation 4000 rpm (6000 x*g*) for 10 minutes. Nuclear extracts were prepared according to the method of Schreiber et al. (1989).

Protein concentrations were determined by measurement of binding of bichincinic acid (BCA) and measurement of absorbance at 405 nm as compared to a standard curve of known concentrations of bovine serum albumin (BSA) protein standards. BCA and BSA standards were purchased from Pierce Chemicals.

Farwestern and immunoblotting

Denaturing polyacrylamide gel electrophoresis and electrophoretic transfer of proteins to either nitrocellulose (MSI, Westboro, MA) or polyvinylidene difluoride

(Immobilon P, Millipore, Bedford, MA) was performed by standard methods (Bollag and Edelstein, 1991). Nonspecific protein interactions were blocked in milk powder (3% wt/vol in Tris buffered saline and 0.1% Tween-20 (TBS-T)) for 30 minutes at room temperature. COS M5 lysates were used at a final concentration of 100 μg/ml (total protein). Affinity purified rabbit anti-cytotoxic cell protease antiserum #5722 (α-CCP), which recognizes a conserved octapeptide in the lymphoid/myeloid granule serine proteinases, was used at a final concentration of 2.5 μg/ml. Horse radish peroxidase conjugated donkey anti-rabbit IgG (Amersham Life Sciences, Oakville, ON) was used at a dilution of 1:1500. The ECL™ (Amersham) detection system was used.

INDUCTION AND MEASUREMENT OF APOPTOSIS

Assessment of nuclear condensation

Nuclear morphology was assessed by staining with the DNA-binding dye 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), which binds in the minor groove of AT-rich regions. Cells were permeabilized for 15 minutes in 0.1% saponin in PBS prior to labeling and incubated for 15 minutes in DAPI at 1 μg/ml in PBS/saponin. Visualization of DAPI stained cells was performed by fluorescence microscopy.

Annexin V-FITC binding measure of membrane asymmetry

Loss of membrane asymmetry was assayed by measurement of annexin V-FITC (Clontech) to live cells in bulk culture by flow cytometry with a Becton-Dickinson FACSCAN, or of cells on microscope slides by fluorescence microcsopy. Cells were not fixed prior to analysis to avoid potential loss of membrane integrity due to fixation. Cells were analyzed within 15 minutes following the addition of annexin V-FITC.

Chromium release assay of cell lysis

Lysis of target cells was assayed by the release of ^{51}Cr into the supernatants of cultured cells (Brunner et al., 1968). Targets were labeled for 1 hour at 37°C in 100 µCi ^{51}Cr (sodium chromate) per 10^6 cells (N.E.N.) and washed 3 times. After treatment with the relevant apoptotic stimulus, either whole CTL, a combination of granule components, or anti-Fas IgM and cells were centrifuged in V-bottom plates for 5 minutes at 600 x *g*. 100 µl of the supernatant was removed for scintillation counting in a Wallac RackGamma II 1270 gamma counter. Percentage of specific release of ^{51}Cr due to the treatment being tested was calculated by [(test - spontaneous)/(total - spontaneous)] x 100.

DNA fragmentation assays

DNA fragmentation in apoptosis cells was measured in three ways: i) ^{3}H-thymidine release assay; ii) standard and pulse field agarose gel electrophoresis and iii) terminal dUTP nick end labeling (TUNEL). Cells were labeled in media containing 1 µCi/ml ^{3}H-thymidine (ICN) at 37°C for 24 hours. After exposure to apoptotic stimuli, cells were lysed in 200 µl of 1% Triton X-100 in Tris-EDTA to solubilize free nucleotide. Percentage of specific ^{3}H-thymidine release is calculated by the equation: [(test - spontaneous)/(total - spontaneous)] x 100, where spontaneous is the measure of release from untreated targets. Cells lysed in 2% sodium dodecylsulfate in 0.2N NaOH were used as a measure of the total ^{3}H-thymidine incorporated into target cell DNA.

Terminal dUTP nick end labeling (TUNEL)

DNA fragmentation was assessed *in situ* by the TUNEL method (Gavrieli et al., 1992). In short, FITC-conjugated dUTP (Boehringer Mannhein Corp.) was added to the free 5'-OH group in the nicks characteristic of the DNA fragmentation observed

in apoptosis. Terminal transferase (BMC), in the presence of the accompanying buffer supplemented with $CoCl_2$ and dATP and incubated at 37°C for 60 minutes, catalyzed the reaction. Cells were permeabilized in PBS/saponin prior to labeling. Cells were visualized by fluorescence microscopy or by confocal laser scanning microscopy (CLSM).

Microinjection of granzyme B into target cells

Cell microinjection was performed on the stage of a Nikon Diaphot inverted microscope using an Eppendorf pressure injector (Model 5246) and micromanipulator (Model 5171). Microinjection needles (approximately 0.1 µm inner diameter) were pulled from glass capillaries using a horizontal electrode puller (Sutter Instrument, Model P-97) and loaded using Eppendorf microloaders. Enzymatically active granzyme B and granzyme B inactivated with anti-granzyme B were diluted in 0.15 M NaCl. To identify injected cells, the injectate contained 0.3% Texas Red Lysine Fixable (Molecular Probes). The concentration of purified human granzyme B was 3.23 mg/ml, which corresponds to 119.4 units/µl. Solutions were injected into the cytoplasm of MCF-7 cells which were plated on glass cellocate cover slips (Eppendorf) 18 hours prior to injection. Medium (RPMI 1640, 10% FBS, 200 µg/ml G418 and 100 µg/ml Hygromycin) was changed before and after injection. Minaschek et al. (1989), using similar equipment demonstrated that the injection parameters used in the present study (pressure: 100 hPa; time 0.5 seconds) delivers approximately 0.05 pL into the cytosol of 3T3 cells. We estimate the volume of MCF-7 cells to be 5 pL, similar to that of 3T3 cells. Thus, intracellular concentrations of the injectate are estimated to be 1% of the pipette concentration, reflecting a 100-fold dilution in the cell.

Immunocytochemistry

Jurkat cells were attached directly to glass microscope slides by centrifugation for 15 seconds at 600 x*g*. COS cells were grown directly onto 0.5 mm glass coverslips. All cells were fixed immediately in paraformaldehyde (2% w/v in PBS) and washed in PBS prior to permeabilization and immunolabeling. Cells were labeled as previously described (Pinkoski et al., 1996) and viewed with a Zeiss fluorescence microscope or by confocal laser scanning microscopy and analyzed with the accompanying CLSM software (CLSM; Leica, Heidelberg). Images were acquired by 32 or 64 line scan averaging using 100x/1.32 N.A. objective under oil immersion. Externalization of phosphatidylserine was measured using FITC-conjugated annexin V (ApoAlert; Clontech) and analyzed by flow cytometry using a Becton Dickinson FACScan.

Adherent cells were grown directly onto glass cover slips and cells from suspension cultures were affixed to glass microscope slides by centrifugation for 15 to 20 seconds at 600 x *g* in a Cytospin centrifuge. Cells were fixed in either 1% formalyn or 2% paraformaldehyde for 15 minutes, washed in PBS for 15 minutes, dried and stored frozen at -20°C until further treatment.

For immunolabeling, cells were permeabilized in saponin (0.1% w/v in PBS) for 15 minutes and blocked in non-fat milk powder (3% w/v in PBS-saponin) for 30 minutes at room temperature (RT). Primary antibodies or antisera were added in blocking solution at a dilution of 1:100 to 1:200 unless otherwise noted. Cells were incubated in the primary antibody for 30-60 minutes at RT, washed three times in PBS-saponin and incubated in fluorophore-conjugated secondary antibody for 30 to 60 minutes at RT Cells were washed three times in PBS-saponin , dried and mounted in 12 µl mounting solution containing *n*-propyl gallate (2.5% w/v) and 85% glycerol in PBS (Giloh and Sedat, 1982). Cells labeled for immunofluorescence were assessed visually within 48 hours.

Protease linked immunoelectron microscopy

EL4 target cells were collected by centrifugation, washed with 0.2 M sodium phosphate (pH 7.2), and fixed in 1% glutaraldehyde. The cells were then washed with phosphate buffered sucrose (6.84% wt/vol), postfixed in OsO_4 (in 0.2 M sodium phosphate, 1.7% sucrose), washed with sucrose-free buffer, dehydrated in ethanol and embedded in Epon 812. Sections 80 to 120 nm thick were mounted on nickel grids for immunocytochemistry studies. Grids were labeled as above for immunofluorescence studies with additional incubations in 10% H_2O_2 for 10 minutes and saturated $NaHIO_4$ for 20 minutes prior to proteinase/antisera labeling. The detection method utilized a gold conjugated (10 nm) goat anti-rabbit Ig (Amersham) at a dilution of 1:5. Prior to examination in the electron microscope, sections were lightly stained with saturated uranyl acetate and lead citrate. Photographs were obtained with a Phillips STEM-420 electron microscope operated at 100 kV.

Immuno- (western-) and far-western blotting

Protein samples were separated by standard discontinuous denaturing polyacrylamide gel electrophoresis (SDS-PAGE) as per Laemmli (1970). After electrophoresis, gels were soaked in transfer buffer (10% acetic acid, 20% methanol) for 15 minutes and transferred to either Nitrobind nitrocellulose (MSI, Westboro, MA) or polyvinylidene difluoride (Immobilon P, Millipore, Bedford, MA) at 250 mA for 30 minutes for proteins under 100 kDa and 60 minutes for proteins larger than 100 kDa in a Tyler semi-dry electroblot apparatus.

Membranes were blocked in non-fat milk powder (5% w/v) in TBS-T (Tris buffered saline with 0.1% v/v Tween-20) for 30 to 60 minutes at RT or 2 hours to overnight at 4°C. Primary antiserum was diluted in blocking solution and incubated for at least one hour at RT or overnight at 4°C. Blots were washed in TBS-T for a total of

30 minutes. Horse radish peroxidase (HRP)-conjugated secondary antiserum (Amersham and Jackson Immunoresearch, Inc.) was used at a dilution of 1:5,000 to 1:15,000 in 5% milk powder in TBS-T. Membranes were incubated in secondary antisera for at least one hour at RT or four hours at 4°C. Blots were then washed for a total of 30 minutes in TBS-T and incubated for 60 seconds in the chemiluminescent substrate ECL (Amersham), blotted lightly and exposed to x-ray film.

Far-western blotting was performed as described above but with the additional step of incubation of blots in protease at a concentration of 100 µg/ml (in TBS) of total protein from lysates of transfected COS M5 cells. Membranes were incubated in protease solution for 2-4 hours at RT prior to incubation in the primary antiserum.

Transfection of COS M5 cells

Granzyme B constructs in the vector pAX142, generated by Dr. A. Caputo, were transfected into COS M5 fibroblasts by standard DEAE-dextran protocol (Caputo et al., 1993). Briefly, COS M5 cells were seeded at 10^6 per 15 cm plate for 16 hours. Plasmid DNA prepared by $CsCl_2$ gradient purification (Sambrook et al., 1989) (4 µg / plate) was added in the presence of DEAE-dextran (50 mg/ml), chloroquine (60 mg/ml) in DMEM (5 ml per plate) for six hours at 37°C. Cells were then treated for 90 seconds in 10% DMSO in PBS supplemented with Ca^{2+} (9 mM) and Mg^{2+} (5 mM) (complete PBS), washed twice in complete PBS, followed by 42 hours in DMEM with penicillin (50 U/ml) and streptomycin (50 µg/ml) at 37°C in 5% CO_2.

Catalytically active granzyme B and inactive pro-enzyme cloned in the vector pAX142 were expressed by transient transfection of COS M5 cells as previously described (Caputo et al., 1993). Enzymatic activity of mature granzyme B was assayed by cleavage of the synthetic substrate *tert*-butyloxycarbonyl-Ala-Ala-Asp-thiobenzylester in the presence of the chromogenic indicator dithiobis(2-nitrobenzoic acid) as described (Caputo et al., 1993).

Immunoprecipitation

Cellular lysates, or subcellular fractions were fixed in 1% formalyn for 30 minutes prior to immunoprecipitation. Sepharose CL-4B protein A resin (Pharmacia Biotech, Baié, PQ) was incubated sequentially with rabbit antiserum #5722 (5 µg/ml), COS M5 lysate containing active granzyme B, zymogen, or granzyme B[S183A] (100 µg/ml) and finally pre-fixed EL4 protein sample. The resin was washed extensively in TBS-T and the protein disassociated following the addition of gel sample buffer containing 2-mercaptoethanol and heating at 100°C for 10 minutes. Immunoprecipitates for HPLC were dissociated from antisera by the addition of 2-mercaptoethanol to 14 mM.

Confocal laser scanning microscopy

Target cells were adhered to poly-L-lysine coated coverslips, fixed in 4% formaldehyde and labeled essentially as noted above for far-western blots with the exception of the addition of saponin (0.1% wt/vol) to permeabilize the cells. In these experiments the detection of labeled proteins was through the use of Texas Red™-conjugated goat anti-rabbit Ig (Jackson Immunoresearch). Cells were counter-stained with FITC-conjugated Con A which labels the endoplasmic reticulum. Jurkat cells were double labeled with granzyme B/α-CCP/Texas Red-conjugated goat anti-rabbit Ig and ANA-N (Sigma) in combination with goat anti-human- conjugated FITC (Jackson Immunoresearch). Labeled cells were viewed with a Leica CLSM™ using a 100x/1.32 N.A. oil immersion objective and analyzed with the accompanying CLSM™ and the Silicon Graphics Imageview software. Images are the result of 64 scan line averaging.

Quantitation of CLSM images

Digital images acquired on the confocal laser scanning microscope were analyzed with the NIH-Image Software to determine the relative level of label corresponding to granzyme B in the cytoplasm and nucleus in TUNEL positive and negative cells. Black and white images were sharpened, edged and thresholded. The Grain Counting Macro was used to count pixels corresponding to immunolabel.

Purification of granzyme, perforin and adenovirus

Human perforin and granzyme B were purified from the human cytolytic cell line YT, as previously described (Hanna et al., 1993; Froelich et al., 1996a). Type 2 adenovirus was purified according to Seth et al. (1986). Murine granzyme B was purified from cytoplasmic granules of the cell line MTL2.8.2 (Pinkoski et al., submitted). Cell pellets were washed in PBS containing 5 mM EGTA, 1 mM $MgCl_2$ and resuspended in 20 ml of the same buffer. A crude cell lysate was obtained by three cycles of freezing and thawing at -70°C and 37°C followed by centrifugation at 12 000 x *g* for 5 minutes at 4°C. The granules in this supernatant were lysed by addition of NaCl to 2 M and freezing and thawing as above. The granule lysate was cleared by centrifugation at 90 000 x *g* for 60 minutes at 4°C and prepared for chromatography on Heparin HiTrap columns (Pharmacia) by ten fold dilution with 50 mM MES pH 6.1. Granule proteins were applied to 1 ml Heparin HiTrap columns which were developed with a linear gradient of 0.5-1.1 M NaCl in 50 mM MES pH 6.1. Granzyme B was eluted at approximately 0.7 M NaCl. Fractions containing granzyme B were detected by hydrolysis of butlyoxycarbonyl-Ala-Ala-Asp-thiobenzyl ester substrate (Caputo et al., 1994). An enzymatically inactive mutant granzyme B[S183A] was expressed using the pAX142 expression vector by transient transfection of COS-M5 cells as described previously (Caputo et al., 1993; Kay and Humphries, 1991). Granzyme B[S183A] was purified by chromatography on Heparin HiTrap followed by HiTrap SP as above.

Fractions containing granzyme B[S183A] were detected by western blotting (Caputo et al., 1994).

Antisera

Antisera against residues 9-16 of granzyme B (provided by Dorothy Hudig, University of Nevada, Reno, Nevada) was generated as previously described (Pinkoski et al., 1996). Rabbit anti-rab4 (clone D-20, polyclonal) and mouse anti-rab5 (clone R28720/L1, monoclonal) were obtained from Santa Cruz Biotechnology, Inc. Secondary antibodies goat anti-rabbit-FITC, goat anti-rabbit-Texas Red™, goat anti-mouse Texas Red™ and mouse anti-rabbit rhodamine were purchased from Jackson Immunoresearch. Anti-Fas IgM was purchased from Upstate Biotechnology, Inc. Gold-conjugated (10 nm particle size) goat anti-rabbit Ig was purchased from Amersham and anti-human nucleolar antigen (autoreactive antiserum) was purchased from Sigma Chemical Corp.

Induction of apoptosis

Granzyme B was added directly to Jurkat target cells at 1 μg/ml in RPMI supplemented with BSA (0.05% w/v). Sublytic doses of perforin was used at 90 U/ml, where one unit is defined in the standard sheep red blood cell hemolytic assay. Cells were washed and resuspended four times in media prior to further treatment (Froelich et al., 1996a). Volumes equivalent to the incubation volume were used for each wash. Infection with Adenovirus (Type 2) was performed in RPMI with BSA (0.05%) as noted above for granzyme B at a multiplicity of infection of 100 plaque forming units per cell. Transiently transfected COS M5 cells were treated with adenovirus or perforin as described above without prior treatment of granzyme B. Inhibitions studies with caspase-3 inhibitor z-DEVD-fluoromethylketone (z-DEVD-

fmk) z-DEVD-fmk (Kamiya, Seattle, WA) were performed by incubating target cells in the presence of the inhibitor (20 μM) for 30 minutes prior to addition of perforin or Ad2. All cells were incubated at 37°C unless otherwise noted. Temperature inhibition studies were conducted by incubating target cells at 4°C, 20°C or 37°C in the presence of granzyme B as described above.

Inhibitor studies

RGD inhibitor peptides GRGDSP and RGDS and negative control peptides GRGESP and GRADSP were obtained from Gibco/BRL-Life Technologies and used at a final concentration of 0.1 mM. Caspase-3 inhibitor, z-DEVD-fmk, was purchased from Kamiya (Seattle, WA) and used at a final concentration of 20 μM. Trafficking inhibitors nigericin (5 μM), bafilomycin A (1 μM), brefeldin A (10 μM), chloroquine (100 μM), mannose (5 mM), mannose 6-phosphate (5 mM) and NH_4Cl (40 mM) were purchased and used at the final concentrations noted. Unless stated otherwise, inhibition studies were performed by incubation of targets in the presence of inhibitor for 30 to 60 minutes prior to the addition of granzyme B. Inhibitors were present during granzyme B incubation.

Acid wash of granzyme B treated target cells

After incubation of target cells in soluble granzyme B at 4°C, 20°C or 37°C, cells were washed three times in ice cold acid-glycine buffer (0.14 M NaCl, 5 mM KCl, 0.5 mM $MgCl_2$-$6H_2O$, 0.68 mM $CaCl_2$-$2H_2O$, 0.1 M glycine; pH 3.0). Following acid wash, cells were washed once in PBS before further treatment with Ad2 or perforin.

CHAPTER III

GRANZYME B BINDS A NUCLEOLAR PROTEIN

CHAPTER III

GRANZYME B BINDS A NUCLEOLAR PROTEIN[1]

INTRODUCTION

Apoptosis induced in target cells by cytotoxic T lymphocytes is believed to occur after CTL-target conjugation which is facilitated by engagement of the T-cell receptor and its accessory molecules (Berke , 1994; Kägi, 1996). This cell-cell contact induces the exocytosis, from the CTL, of granules which contain the potential cytolytic effector molecules. These include the pore forming protein perforin (cytolysin) and a family of serine proteases collectively known as granzymes. The series of events that occur after degranulation, which lead to target cell death, remain to be elucidated.

Perforin is believed to create pores in the target cell membrane which may disrupt osmoregulation and facilitate transfer of the granzymes. Once the granzymes enter a target cell, it is hypothesized that cleavage of their respective substrates results in cell death (Podack et al., 1991). Hallmarks of CTL-induced cell death include many of the signs of apoptosis: DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage (Duke et al., 1983). Granzyme B has been implicated in a number of these processes, most notably with the fragmentation of DNA.

Granzyme B is the prototypic member of the granzyme family of proteases (Lobe et al., 1986) and is abundantly expressed in response to a diverse array of CTL

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stimuli: αCD3, concanavalin A (Con A), phorbol myristate acetate (PMA) and allogeneic stimulation (Prendergast et al., 1992). Expression of granzyme B correlates with lytic activity of CTL *in vitro* (Prendergast et al., 1992) and with the activity of infiltrating T lymphocytes in allografts *in vivo* (Mueller et al., 1988). Granzyme B has also been isolated as the activity known as fragmentin (Shi et al., 1992a). This activity is necessary for degradation of DNA in target cells by CTL. A role for granzyme B in the degradation of target cell DNA is further supported by the granzyme B homozygous null mutant mice (Heusel et al., 1994). These mice have a severely depressed ability to cause rapid DNA fragmentation in target cells.

Granzyme B has an uncommon substrate specificity of aspartic acid at P1 (Murphy et al., 1988; Poe et al., 1991). This preference is also observed in the caspase family of proteases, including *ced* 3 (Xue et al., 1995) and interleukin 1β-converting enzyme (ICE) (Thornberry et al., 1992). These molecules play a pivotal role in apoptosis in *C. elegans* and higher eukaryotes respectively. Physiological substrates of this group of enzymes with the rare substrate specificity of Asp at P1 will undoubtedly provide insights into the respective apoptotic processes in which they are involved.

In order to understand how granzyme B is involved in mechanisms of target cell destruction it is necessary to identify cellular substrates in the target cell. As a primary approach we undertook to demonstrate that granzyme B, given access to a permeabilized target cell *in situ* , could bind to potential substrates. We have developed an *in situ* binding assay using permeabilized cells and recombinant granzyme B that allows us to visualize potential substrates after immunostaining with anti-granzyme B antiserum. In this manner we can determine if granzyme B binds to intracellular membrane proteins and to further localize potential sites of action of the granzyme.

Confocal laser scanning microscopy (CLSM) and immunoelectron microscopic analyses demonstrate that granzyme B recognizes a nuclear substrate. The labeling pattern observed corresponds with regions of positive staining with uranyl acetate which binds to heterochromatin in the nucleus. Positive labeling of target cells with granzyme B is dependent on the presence of a catalytically active proteinase, since an inactive proenzyme form of granzyme B fails to give rise to any binding in the target cells. Far-western blotting and immunoprecipitation of subcellular fractions of target cells have shown that the putative substrate of catalytically active granzyme B is an 80k nuclear protein. Minor cytosolic bands of 50 and 94k are also observed. A cytoplasmic band of 69k is detected by both active and zymogen forms of granzyme B.

RESULTS

Granzyme B recognizes proteins in the nucleus and cytoplasm of target cells

Granzyme B was expressed in COS M5 by transient transfection. Enzymatic activity was assayed by the ability to cleave the synthetic substrate Boc-Ala-Ala-Asp-thiolbenzyl ester. The pro-enzyme form of granzyme B was expressed in the same manner and does not possess catalytic activity.

Granzyme B, purified from transfected COS cells, was used in a far-western blot to screen for potential substrates. Protein blots of whole cell lysates and subcellular fractions of target cell protein were analyzed in this manner. Figure 3.1 shows that granzyme B binds to a nuclear protein of M_r = 80,000 in all cell lines examined. This protein is not recognized by inactive granzyme B zymogen, which differs from the mature proteinase by an additional dipeptide (Gly-Glu) at the N-terminus. Minor cytosolic proteins of M_r = 50,000 and 94,000 are also detectable (Figure 3.2). A cytoplasmic protein of M_r = 69,000 is recognized by both active and zymogen forms of granzyme B (Figure 3.2).

Figure 3.1 Far-western blot of nuclear extracts labeled with granzyme B and zymogen. Electroblot of nuclear extracts (50 μg/lane) labeled sequentially with granzyme B or zymogen, α-CCP and donkey anti-rabbit IgG-horse-radish peroxidase. Detection is a result of chemiluminescence with ECL. A single band of approximately 80k is detected in all cell lines tested. Blots were probed first with zymogen, stripped and re-screened with active granzyme B.

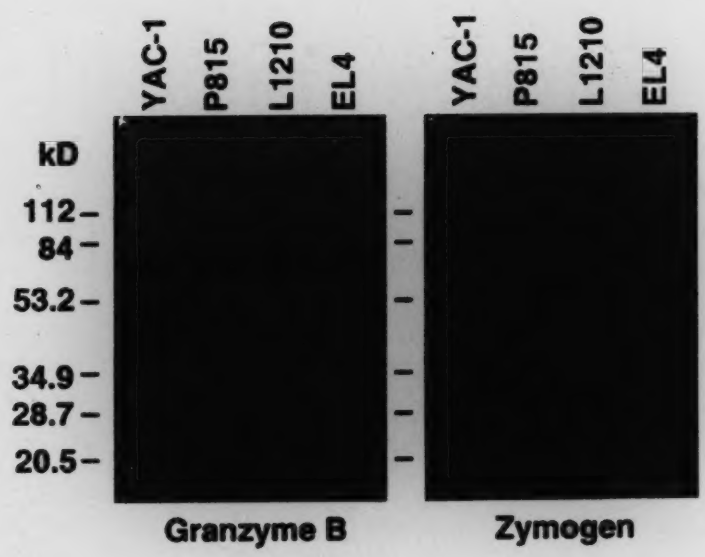
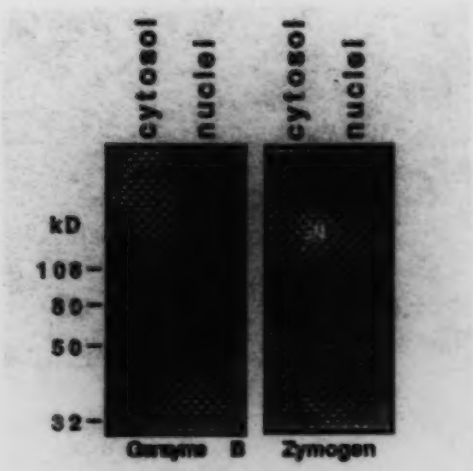

YAC-1
P815
L1210
EL4
YAC-1
P815
L1210
EL4
kD
112
84
53.2
34.9
28.7
20.5
Granzyme B
Zymogen

Figure 3.2 Far-western blot of EL4 sub-cellular fractions labeled with granzyme B or zymogen. Each lane contains 50 μg of the indicated protein lysate. Detection is by chemiluminesence as in Figure 1. A nuclear band of 80 kDa is detected with active granzyme B, as are faint cytosolic bands of 50 and 94 kDa. This signal is dependent on the addition of COS M5 lysate containing either of these granzyme B species. Screening with α-CCP antiserum alone does not give rise to any 69 kDa bands (Caputo et al., 1993). Granzyme B and zymogen both recognize a cytoplasmic protein of 69 kDa.


cytosol
nuclei
cytosol
nuclei
kD
108
80
50
32
Granzyme B
Zymogen

These observations are supported by proteinase-linked immunoprecipitation of target cell proteins. EL*4* proteins were fixed in 1% formaldehyde prior to incubation with granzyme B and precipitated with α-CCP antiserum and protein A-Sepharose. Figure 3.3 shows an 80kDa nuclear protein and 50, 69 and 94kDa cytoplasmic proteins following purification by protease-linked immunoprecipitation. Each band was subsequently isolated by elution from SDS-PAGE. Treatment of the target cell proteins with formaldehyde is a necessary measure to ensure the precipitation of these proteins, but renders the proteins inaccessible to the sequencing chemistry.

Subcellular localization of potential granzyme B substrates

An approach similar to that used in the far-western blot analysis was taken to determine the intracellular localization of proteins recognized by granzyme B. Target cells affixed to poly-L-lysine coated coverslips were fixed in formaldehyde and then incubated in the presence of granzyme B or zymogen, α-CCP antiserum and Texas Red-conjugated goat anti-rabbit IgG. Cells were also labeled with FITC-conjugated Con A. Con A labels the endoplasmic reticulum and provides a reference of cellular morphology.

In all cell lines examined, approximately 50 to 80% of cells showed uptake of label and all cells that labeled displayed similar patterns. Figure 3.4 shows representative cells labeled with granzyme B or zymogen. P815 target cells also show a nuclear labeling with granzyme B. However, this pattern appears to be heavily concentrated in the nucleolus (Figure 3.4, panels C and E). Endogenous serine proteinases can be detected with the α-CCP antiserum in a cytoplasmic granular distribution, but this labeling pattern is not shown in order to highlight the proteinase-specific nucleolar labeling of P815 as a target cell. Permeabilization of cells with 0.1%

Figure 3.3 Proteinase-linked immunoprecipitation of ^{35}S-Met labeled EL4 protein lysates. Nuclear and cytoplasmic fractions were incubated sequentially in 1% formaldehyde and COS M5 lysate containing active granzyme B followed by precipitation with α-CCP and Sepharose CL-4B-conjugated protein A. Following immunoprecipitation, individual proteins were purified by elution from SDS-PAGE. A nuclear protein of 80k is precipitated (lane A) as well as cytoplasmic proteins of 94, 69 and 50k (lanes B-D respectively). The 69k band is also precipitable with inactive granzyme B zymogen.

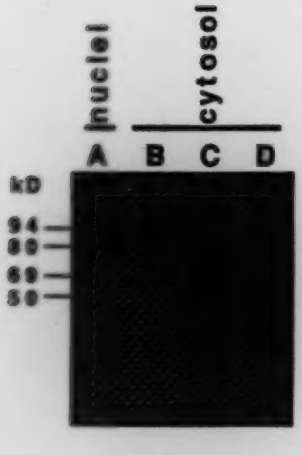
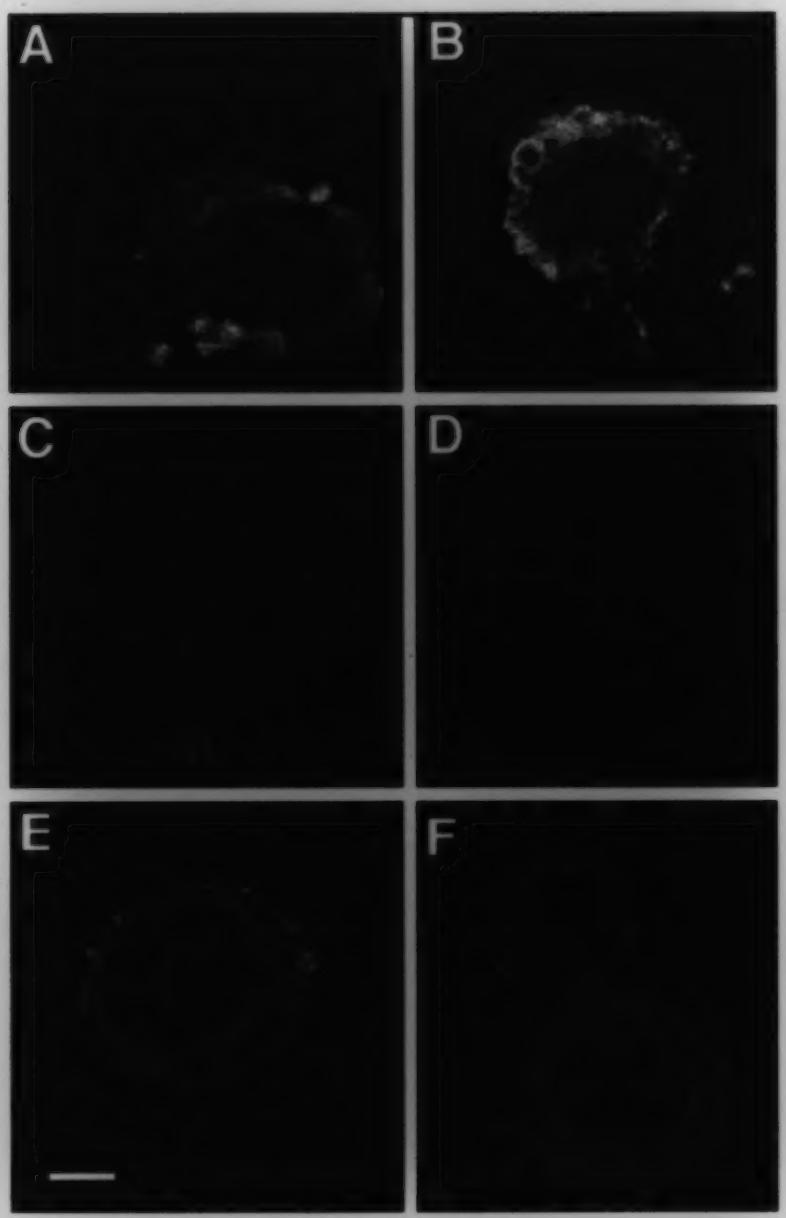

nuclei
cytosol
A
B
C
D
kD
94
68
43
30

Figure 3.4 *In situ* proteinase-linked immunocytochemical analysis of granzyme B binding in target cells. EL*4* (panels A and B) L1210 (panels C and D) and P815 (panels E and F) were labeled with granzyme B (panels A, C and E) or zymogen (panels (B, D and F) followed by α-CCP and Texas Red-conjugated goat anti-rabbit IgG. All panels show cells counter-stained with FITC-conjugated Con A. Cells were viewed by CLSM. Scale bar is 5 μm.


A
B
C
D
E
F

saponin was necessary to observe any signal arising from the addition of granzyme B or α-CCP.

Granzyme B gives rise to a staining patterns in EL4, L1210 (Figure 3.4, panel C), and Jurkat (Figure 3.5) that is granular throughout the nucleoplasm. This staining is consistent with nucleolar patterns observed in multinucleolar cell types and lines (Fakan and Hernandez-Verdun, 1986; Vandelaer et al., 1993; Scheer and Weisenberger, 1994). Labeling of these cells with granzyme B is observed throughout the cytoplasm, but cannot be assigned to a specific sub-cytoplasmic localization. Closer scrutiny of localization of granzyme B binding proteins in EL4 cells is described in immunoelectron microscopic studies below.

Double label of Jurkat cells with the nucleolar specific antiserum ANA-N and granzyme B shows a co-localization pattern. In these experiments granzyme B binding appears red and the nucleolus is labeled green. Where the two patterns overlap the label appears yellow. Figure 3.5 shows that murine granzyme B gives rise to a nucleolar labeling pattern in the human Jurkat target cell line. The Jurkat nucleoli appear green when labeled with the inactive zymogen, due to a positive green signal from the ANA-N and a lack of a red signal from granzyme B zymogen.

Interaction between granzyme B and potential substrates requires an open catalytic site

Non-specific electrostatic interactions between proteinase and the proteins detected are ruled out by the absence of binding by the inactive proenzyme. This proenzyme differs from the active proteinase by only an additional two amino acid residues (Gly-Glu) at the N-terminus. Removal of this dipeptide is necessary for the mature protein to yield enzymatic activity (Caputo et al., 1993).

The importance of the negative control utilizing the zymogen granzyme includes other relevant negative controls for successful application of this system. Positive

Figure 3.5 Nucleolar localization of granzyme B label in target cells. Jurkat cells were labeled with granzyme B (panel A) or zymogen (panel B) as described in Figure 3.4, but using the nucleolar specific antisera ANA-N (FITC) as the secondary label. Granzyme B label appears red, ANA-N appears green and coincidental label appears yellow. Cells were viewed by CLSM. Scale bar is 5 μm.

A
B

signals caused by interaction of the antisera binding directly to antigens in the target cells are ruled out, as well as any signals that may have arisen from the interaction of any of the secondary labeled antibodies to target cell proteins.

We first performed the farwestern blots with either active enzyme or zymogen. This was followed by stripping of the blots and re-screening with zymogen or active enzyme respectively. This removed the possibility that the stripping process adversely affected the ability of either active enzyme or zymogen to bind proteins in this farwestern blot arrangement.

An additional measure to demonstrate that the positive signals attributed to binding of catalytically active granzyme B also represents a specific interaction between the α-CCP antiserum and the granzyme B from the COS lysate is the observation that pre-incubation of this antiserum with a BSA conjugated octapeptide (the peptide that served as the original immunogen) successfully competes out binding of the antiserum to granzyme B COS lysate labeled target cells (data not shown).

Nuclear granzyme B binding protein(s) co-localizes with heterochromatin

EL4 target cells were fixed, embedded and sectioned onto nickel grids. Sections were labeled according to the scheme outlined above for immunofluorescence analyses. The detection method utilized a gold-conjugated anti-rabbit IgG to detect the α-CCP antiserum. Representative cells labeled with granzyme B or zymogen are shown in Figures 3.6 and 3.7 respectively. Granzyme B gives rise to a nuclear staining that uniformly co-localizes with the heterochromatin. Heterochromatin binds greater amounts of the counter-stain uranyl acetate and stains darker than euchromatin.

Cytoplasmic labeling by granzyme B observed by CLSM is not observed by electron microscopy. The glutaraldehyde fixative used for electron microscopy masks

Figure 3.6 Proteinase-linked immunoelectron microscopic analysis of granzyme B label of target cells. Ultra-thin sections of EL4 target cells were labeled with granzyme B in combination with α-CCP and goat anti-rabbit Ig gold conjugate (10 nm particle). Sections were counter-stained with uranyl acetate and lead citrate. 24,000x magnification.

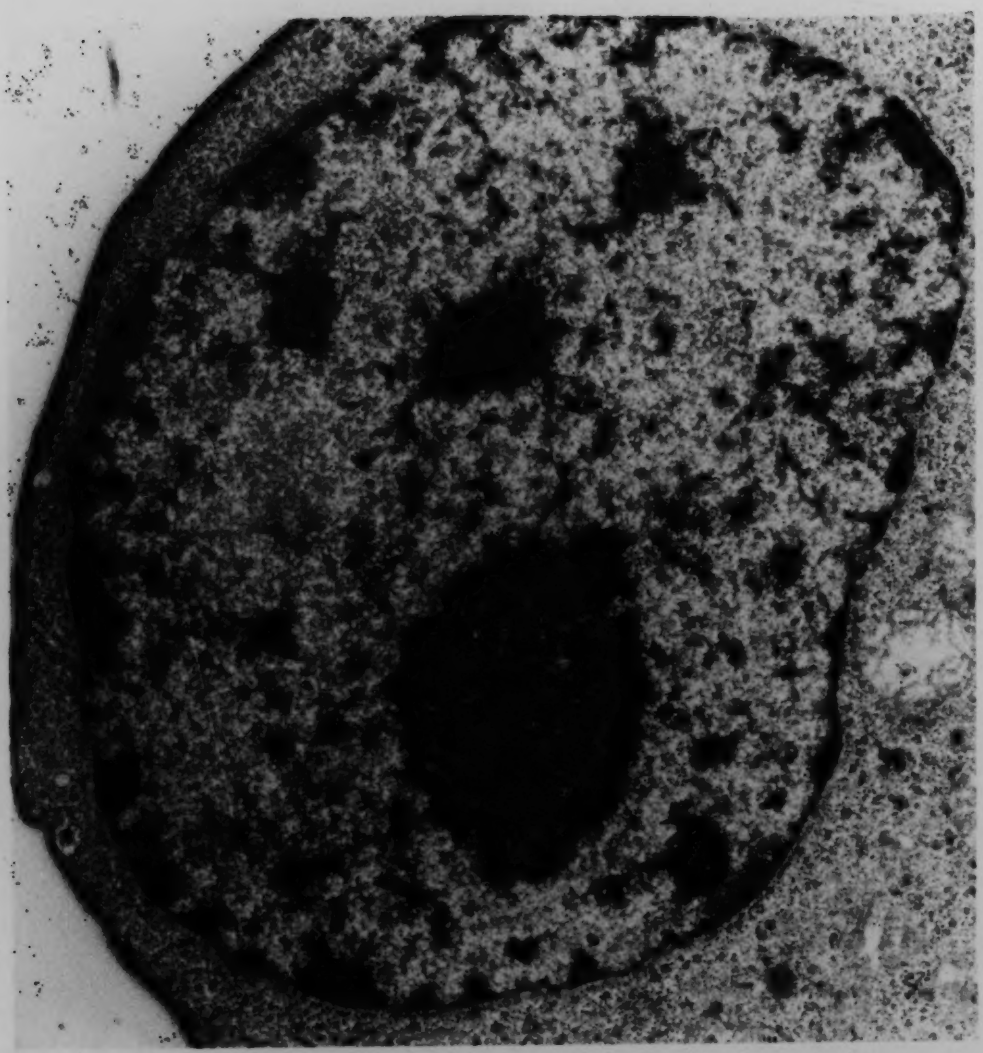
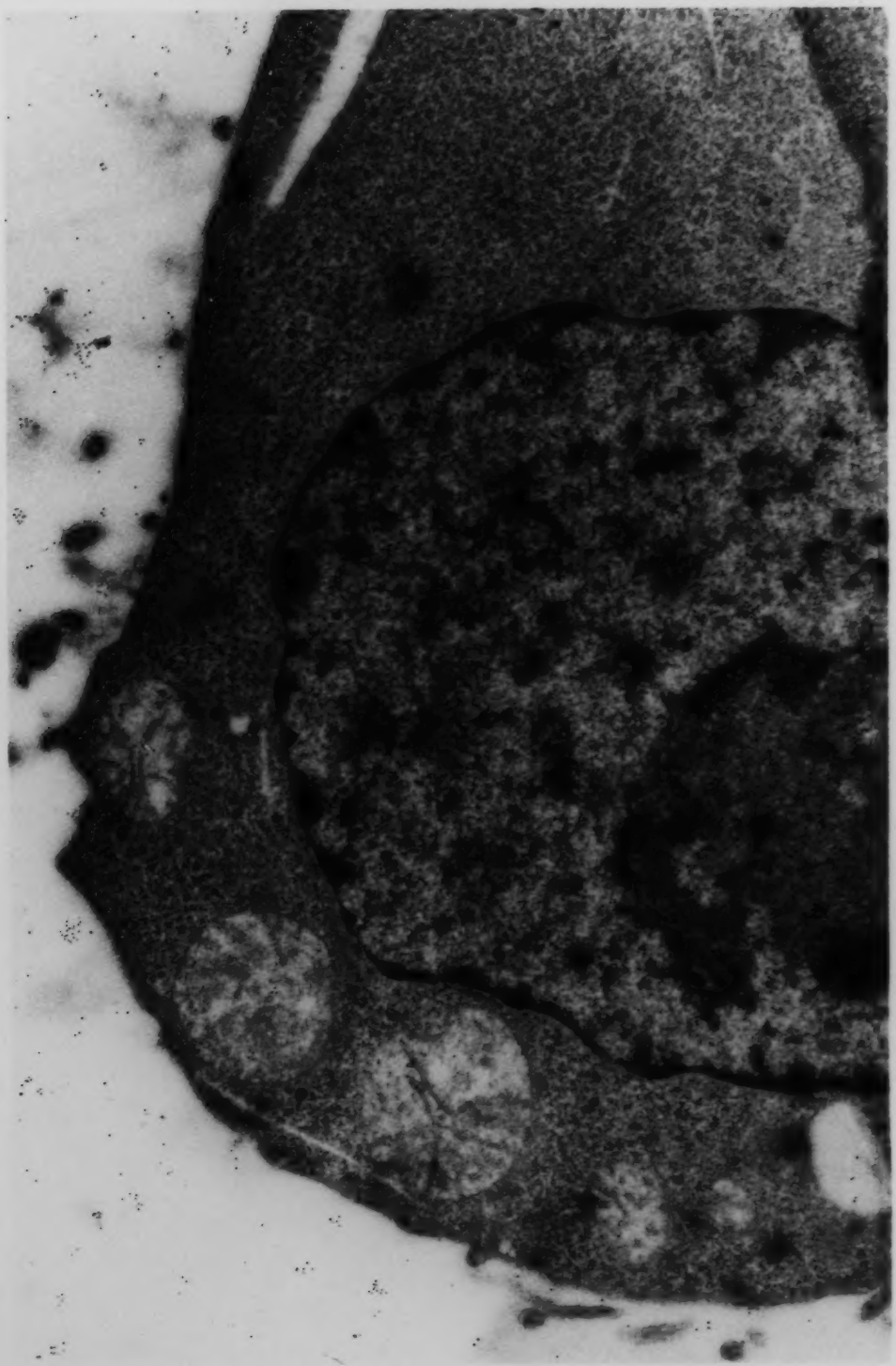


Figure 3.7 Proteinase-linked immunoelectron microscopic analysis of granzyme B label of target cells. Ultra-thin sections of EL4 target cells were labeled with granzyme B zymogen in combination with α-CCP and goat anti-rabbit Ig gold conjugate (10 nm particle). Sections were counter-stained with uranyl acetate and lead citrate. 24,000x magnification.



the binding of granzyme B to any potential cytoplasmic substrates. This masking effect was also observed when glutaraldehyde-fixed cells were viewed by confocal microscopy.

DISCUSSION

Far-western blot analysis of EL4 subcellular fractions demonstrate that catalytically active granzyme B binds to a nuclear protein with an apparent molecular weight of 80k and cytosolic proteins of 50 and 94kDa that are not detected by inactive zymogen. This suggests that the interaction of granzyme B with this proteins is dependent on an active catalytic site within the proteinase facilitated through an open specificity pocket. A cytoplasmic protein of 69k is recognized by both active and inactive forms of granzyme B. The possibility of a relationship between these proteins has not been examined. Granzyme B does not contain any known linear or bipartite nuclear localization signal, so the possibility exists that granzyme B requires a cytoplasmic protein to shuttle the proteinase into the nucleus. Perhaps the 69k protein fills this role since a shuttle protein is not likely to be not dependent on an active catalytic site in granzyme B.

Nuclear labeling with granzyme B shows a punctate staining pattern throughout the nucleus and is heavily concentrated surrounding or within the nucleolus. The nature of the nuclear labeling is dependent on the target cell line. Given that granzyme B recognizes 80k nuclear proteins in all of the cell lines tested, this is likely to reflect differences in the nucleolar composition and distribution between cell lines rather than differences in the substrate(s) being recognized.

A more detailed nuclear labeling pattern was observed through protease-linked immunoelectron microscopy. These studies demonstrate that this nuclear granzyme B-binding protein co-localizes with the heterochromatin. This chromatin fraction contains transcriptionally inactive DNA, including the centromere which provides a link to the

structural framework of the nuclear matrix. Also contained in the heterochromatin is DNA that is merely transiently transcriptionally repressed. The heterochromatin labeling of target cells with granzyme B is not likely to have arisen from recognition of a centromere-associated protein. Distribution of the centromere gives rise to a speckled staining pattern throughout the nucleus (Weimer et al., 1992) and is markedly different from all patterns observed with granzyme B labeling.

One of the hallmarks of apoptosis is nucleolar disintegration (Kerr et al., 1972) followed by DNA fragmentation and chromatin condensation (Duke et al., 1983). Based on the data presented here, a hypothesis suggests that granzyme B may cleave a substrate that is responsible for maintaining the condensation of heterochromatin. Such a cleavage may then result in the exposure of a DNase sensitive site or motif that would otherwise be sequestered. In this model granzyme B would bring about the destruction of a target cell by rendering the target cell sensitive to destructive machinery that is constituitively present and active in the target cell.

These studies demonstrate that enzymatically active granzyme B can be used to successfully locate proteins within a target cell that are bound by granzyme B in a manner resembling proteinase-substrate interactions. Although granzyme A has been demonstrated to bind nucleolin in target cell lysates (Pasternack et al., 1991), this is the first example of a granule serine proteinase from a CTL binding to proteins within a target cell *in situ*. The labeling pattern and the size of the proteins detected suggest that the 80k protein recognized by granzyme B does not correspond to nucleolin (110k) (Lischwe et al., 1982) which, although found in the nucleolus, displays a different nuclear labeling pattern (Biggiogera et al., 1989, 1990).

Granzyme B has been demonstrated to bind to two proteins in target cells. While transfer of granule proteinases from a CTL to its respective target cell remains to be visually demonstrated, these data show that granzyme B can recognize at least two cellular proteins in the target cell. It has recently been shown granzyme B can cleave

CPP32, caspase-3 (Darmon et al., 1995). Failure to detect caspase-3 in farwestern blots is likely due to the low abundance of caspase-3. Given the lack of sensitivity of the assay described here, the 50, 69, 80 and 94 kDa proteins are likely present in significant abundance.

Since the time period between the initial contact between CTL and target cell and ultimate destruction of the target cell is as low as six minutes (Perez et al., 1985), it is quite likely that the effects of the CTL are mediated through a pathway that utilizes existing components within the target. Included in this observation is the probability that substrates of granzyme B are widely and constituitively expressed in all cell types that are sensitive to destruction by CTL. This hypothesis is supported by the observation of an 80 kDa granzyme B-reactive protein in a diverse panel of cell lines. The binding of granzyme B to these proteins may represent a proteolytic cleavage event that results in either the release of a destructive activity or renders some vital sequestered motif accessible to pre-existing cellular degradative processes.

CHAPTER IV

AUTONOMOUS ENTRY OF GRANZYME B INTO TARGET CELLS

CHAPTER IV

AUTONOMOUS ENTRY OF GRANZYME B INTO TARGET CELLS[2]

INTRODUCTION

Cytotoxic T lymphocytes (CTL) are the effectors in cell-mediated immunity that destroy virus infected and neoplastically transformed cells (for reviews see Atkinson and Bleackley, 1995; Berke, 1994, 1995; Kägi et al., 1996). Following adhesion of CTL and target cell, the plasma membranes interdigitate and cytolytic granules migrate toward the site of contact releasing their contents into the apposed space. This secretory process forms the basis of the lethal hit model of granule mediated cytotoxicity (Henkart, 1985) in which it is postulated that the molecules contained within the cytoplasmic granules are the lytic effectors that act in concert to bring about target cell apoptosis.

A number of proteins have been isolated and characterized from cytolytic granules. The pore-forming protein, perforin (Masson and Tschopp, 1985), is a granule protein that is believed to insert into the membrane of the target cell, providing a channel that facilitates the entry of other granule components into the cytoplasm of the target cell (reviewed by Liu et al., 1995). Granzyme B, also known as cytotoxic cell proteinase 1 (CCP1; Lobe et al, 1986) or fragmentin 2 (Shi et al., 1992b), is the prototypic member of the family of serine proteinases that reside in the cytolytic granules of CTL, natural killer (NK) and lymphokine activated killer (LAK) cells (Lobe

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[2] A version of this chapter has been submitted for publication to the Journal of Cell Biology.

et al., 1986; Masson and Tschopp, 1987), collectively termed granzymes. While perforin alone causes necrotic cell death by osmolysis, apoptosis is mediated by the concerted actions of perforin and the granzymes.

Although there has been no direct demonstration of transfer of molecules from CTL to target, a number of laboratories have provided evidence for the requirement of granule proteins in stimulating apoptosis in target cells. Granzyme B and perforin have been shown to bring about rapid DNA fragmentation in target cells by a mechanism that is attributed to granzyme activity (Shi, et al., 1992a, 1992b). Similarly, transfection of the rat basophilic cell line, RBL, with perforin and granzyme renders the RBL capable of cytolytic activity when directed against a number of target cell lines (Shiver, et al., 1992; Nakajima and Henkart, 1994). Recently, conclusive evidence for a role of granzymes in target cell apoptosis has come from the generation of granzyme B deficient mice. The CTL of granyzme B-/- mice display a severely reduced ability to induce rapid DNA fragmentation in target cells (Heusel et al., 1994; Shresta et al., 1995).

In support of the notion that the granzymes induce apoptosis, in part, by cleaving cytosolic proteins, granzyme B has been reported to activate certain ICE/CED-3 cysteine proteinases, or caspases (Alnemri et al., 1996), that are closely linked to apoptotic cell death (reviewed by Martin et al., 1995a; Nagata, 1997), including caspase-3 (Darmon et al., 1995), caspase-7 (Gu et al., 1996; Chinnaiyan et al., 1996). Caspase-3 (CPP32), is processed in murine targets treated with CTL (Darmon et al., 1995) and targets exposed to granzyme B and perforin contain processed caspase-1 (ICE) (Shi et al., 1996), caspase-3 and -7 (Chinnaiyan et al., 1996). Thus, it is implied that granzyme B gains access to the interior of the target cell where it can cleave and activate these substrates. Activation of the caspases has been shown to be essential in DNA fragmentation (Darmon et al., 1996) and more recently for externalization of phosphatidylserine in the plasma membrane of cells undergoing apoptosis (Martin et

al., 1996). Activated caspase-3 cleaves and activates a DNA fragmentation factor (DFF; Liu et al., 1997) and inactivates a number of DNA repair enzymes, including poly ADP-(ribose)polymerase (PARP; Nicholson et al., 1995; Tewari et al., 1995) and DNA dependent protein kinase (Song et al., 1996a, 1996b).

In addition to the cytoplasmic substrates of granzyme B there is an increasing body of evidence suggesting that granzyme B may act in the nucleus. In two independent studies, granzyme B was shown to bind to nuclear proteins (Pinkoski et al., 1996; Trapani et al., 1996). At least one of these potential substrates is associated with heterochromatin and the perinucleolar region of the nucleus (Pinkoski et al., 1996).

Despite major advances in our understanding of mechanisms of granule-mediated CTL killing, there has been no direct demonstration of transfer of molecules from cytolytic granules to target cells and no information on the resultant trafficking of CTL proteins once they have gained entry into the target cell. It was recently shown that granzyme B internalization is facilitated by perforin or a replication deficient adenovirus and that both signals are required for target cells lysis (Froelich et al., 1996a).

In order to define the intracellular pathway taken by granzyme B after it is released from the granule of the CTL, we have utilized an in vitro system to track the movement of the granzyme within a target cell. In the present study we demonstrate that granzyme B can enter a target cell independently of perforin, and once internalized, it resides in endosomes. This intracellular sequestration prevents the granzyme from gaining access to its substrates and thus cell death does not occur. In contrast, we show that direct injection of granzyme B into the cytoplasm induces cell death directly. A second stimulus is required to initiate apoptosis, which involves release of granzyme from the endosome and its translocation to the nucleus. Detection of granzyme B in the nucleus of target cells is associated with early onset of DNA fragmentation.

In the widely accepted model of granule mediated killing by cytotoxic lymphocytes, granzyme B entry into the target cell is facilitated by the pore forming molecule, perforin. Here we demonstrate that granzyme B is readily internalized into endocytic vesicles independently of perforin. However, induction of the lytic pathway requires a second signal that can be provided by perforin or adenovirus. This results in the release of granzyme B into the cytoplasm and subsequent apoptosis of the target.

RESULTS

Granzyme B is internalized by target cells independently of perforin

Jurkat target cells were incubated in media containing soluble granzyme B for 30, 60 and 120 minutes. After washing they were treated with anti-granzyme antiserum, followed by a Texas Red-conjugated secondary antibody and analyzed by confocal laser scanning microscopy (CLSM). At 30 minutes there appeared to be an accumulation of granzyme B at the plasma membrane as well as in the cytoplasm (Figure 4.1, panel B). Labeling at the plasma membrane was reduced at subsequent time points and the cytoplasmic pattern became predominant. After two hours, the granzyme had a peri-nuclear distribution, but did not appear to enter the nucleus (Figure 4.1, panel D).

We also studied the ability of granzyme B alone to trigger apoptosis in target cells. Jurkat, treated with granzyme B, were labeled by the terminal deoxynucleotidyltranferase nick end labeling (TUNEL) technique to assess DNA fragmentation, and analyzed by CLSM. These experiments were also performed in YAC-1 cells with analysis of nuclear morphology, after 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, by fluorescence microscopy. Cells labeled by either TUNEL or DAPI were immunolabeled to detect granzyme B. As shown in Figure 4.2 (Panels A and E), we did not observe DNA fragmentation or nuclear condensation under these conditions and the cells appeared entirely normal. These results clearly

Figure 4.1 Granzyme B is internalized by target cells independently of perforin. Jurkat target cells were treated with soluble granzyme B for 0, 30, 60 and 120 minutes at 37°C (a) - (d) respectively. Cells were fixed in 2% paraformaldehyde prior to immunolabeling with anti-granzyme antiserum and staining with Texas Red conjugated goat anti-rabbit antibody. Labeled cells were viewed by confocal laser scanning microscopy. Scale bar is 10 μm.

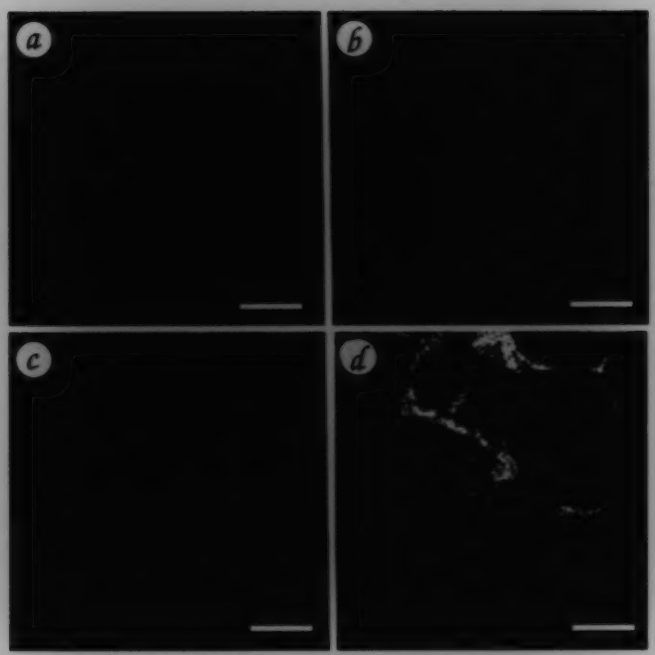
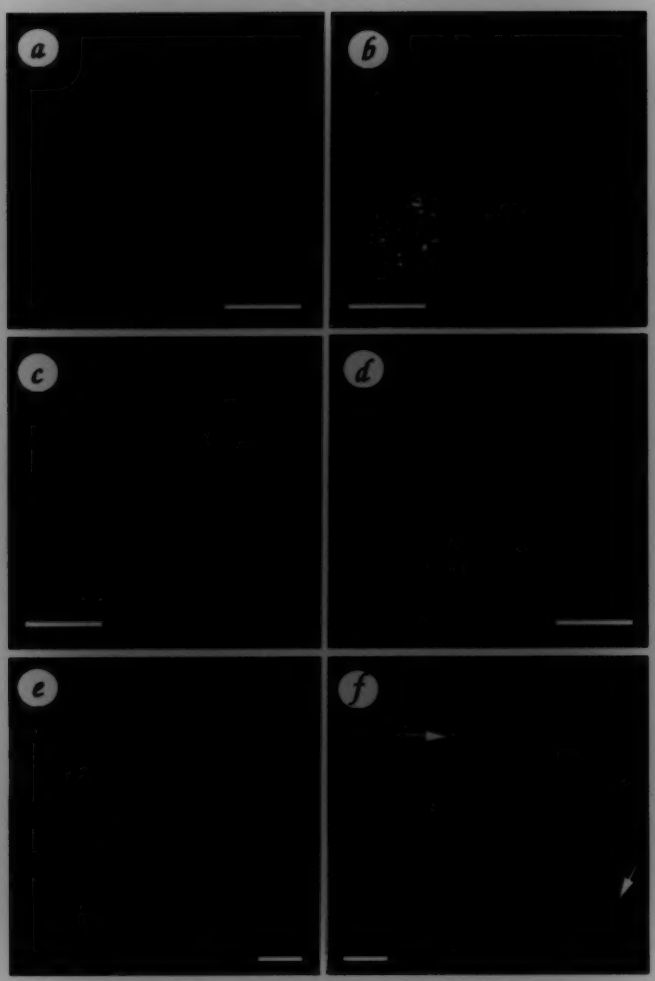

a
b
c
d

Figure 4.2 Target cells that have internalized granzyme B undergo apoptosis only after exposure to perforin. Jurkat target cells were treated with granzyme B, washed to remove soluble granzyme and incubated in sublytic levels of perforin (a)-(d). Cells were labeled for DNA fragmentation by the TUNEL protocol with dUTP-FITC. Panels (a)-(d) show the progression of DNA fragmentation observed in apoptotic death induced by granzyme B and perforin from a healthy cell (a); early DNA fragmentation observed at early time points (b); an apoptotic cell with considerable DNA fragmentation and reduced nuclear size (c); and advanced DNA fragmentation and severe nuclear condensation (f). YAC-1 cells were treated with murine granzyme B, washed and stimulated with buffer (e) or sublytic levels of perforin (f). DNA of permeabilized cells was stained with DAPI and the cells viewed by fluorescence microscopy. Examples of condensed nuclei of apoptotic cells are highlighted by arrows. Scale bar is 10 μm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demonstrated that granzyme B enters target cells independently of other components of the cytolytic granules of CTL, most notably perforin. Furthermore, despite the accumulation of granzyme B within target cells, we failed to observe DNA fragmentation or nuclear condensation, two hallmarks of apoptosis.

Target cells that have internalized granzyme B undergo apoptosis only after exposure to perforin or Ad2

First, Jurkat and YAC-1 target cells were treated concurrently with granzyme B and sublytic doses of perforin. As revealed by TUNEL label and DAPI stain, this resulted in rapid DNA fragmentation and nuclear condensation within 30 minutes (Figure 4.2). Then targets were first incubated in media containing granzyme B for 60 minutes and washed extensively before being treated with sublytic doses of perforin. Cells treated in this two step sequential manner also showed significant DNA fragmentation (Figure 4.2, panels B-D), severe nuclear condensation and a drastic reduction in size (Figure 4.2, panel F). This was in stark contrast to the results seen with cells exposed to granzyme B alone (Figure 4.2, panels A and E). Although perforin is dispensable for the internalization of granzyme B, it is absolutely required to deliver a death signal to target cells containing granzyme B.

Adenovirus can effectively deliver proteins into cells, and triggers apoptosis in cells treated with granzyme B (Froelich et al., 1996a). Thus, we pre-treated Jurkat with granzyme B for 60 minutes, washed the cells extensively and followed with infection by a replication deficient strain of Ad2. TUNEL labeled cells were visualized by CLSM (see Figure 4.1, panel B). As we observed with perforin, pretreatment with granzyme B rendered the cells sensitive to the induction of apoptosis by the secondary stimulus, in this case the virus. Infection by Ad2 in the absence of granzyme B had no apparent deleterious effects on the target cells. Therefore, Ad2 and perforin appear to function in a similar manner to initiate apoptosis in cells pre-loaded with granzyme B.

Microinjection of granzyme B into the cytoplasm of target cells results in cell death

Granzyme B was injected directly into the cytoplasm of MCF-7 cells, an adherent breast carcinoma cell line. Granzyme B induced apoptosis in MCF-7 cells in a dose-dependent fashion (Table 4.1). Introduction of approximately 80 fg/cell (intracellular concentration of approximately 500 nM) granzyme B led to apoptosis in 40% of cells in two hours. Staining of injected cells with Hoescht showed condensed nuclei, a hallmark of apoptotic cell death (data not shown). Apoptosis was due to the enzymatic activity of granzyme B, since an equivalent amount of granzyme that had been inactivated with anti-granzyme B did not induce apoptosis.

Granzyme B is transported through an endocytic pathway.

Granzyme B internalized by cellular means did not result in cell death, however granzyme B injected directly into the cytoplasm of a target cell did result in apoptosis. In order to explain this discrepancy we undertook to investigate the possibility that the granzyme was being internalized and sequestered in a cytoplasmic vesicle. As shown earlier, the ability of granzyme B to induce apoptosis is profoundly influenced by the presence of Ad2. Infection with Ad2 occurs via receptor mediated endocytosis, internalization into early endosomes and subsequent penetration of the virus into the cytoplasm (Svensson and Persson, 1984; Seth et al., 1984, 1986; Varga et al., 1991; Greber et al., 1993). We therefore investigated whether granzyme B internalization involved a similar pathway by co-localization studies with an endosomal marker.

Small Ras-like proteins of the Rab family are known to be involved in vesicular trafficking (Chavrier et al., 1990; Novick and Brennwald, 1993). Rab4 has been identified on endocytic vesicles that recycle plasma membrane constituents from early

Granzyme B units / cell	fg / cell	nM / cell	% apoptosis	death / total
1×10^{-6}	27	179.4	3.34 +/- 0.51	19/559[a]
2×10^{-6}	54	358.9	28.41+/- 21.01	17/684[b]
3×10^{-6}	81	538.3	41.32 +/- 3.08	233/485[b]
4×10^{-6}	108	717.8	59.09	166/286
Inactive granzyme B				
3×10^{-6}	81	538.3	0.00 +/- 0.00	0/587[a]

Table 4.1 Granzyme B, but not inactivated granzyme B, induced cell death by direct injection into the cytoplasm of MCF-7 cells in two hours. Percent apoptosis represents number of dead cells divided by the total injected cells and then subtracted percent death of control cells which were injected only with 0.3% Texas Red and 0.15M NaCl. a and b represent the total number of cells from two and three experiments respectively.

endosomes (Schmid et al., 1988; van der Sluijs et al., 1991, 1992; Bottger et al., 1996). In order to determine whether granzyme B was internalized into such a vesicle, co-localization studies were performed with the granzyme and rab4. Jurkat target cells were incubated in media containing purified granzyme B for one hour to allow uptake. Figures 4.3 and 4.4 show representative Jurkat and HeLa cells, loaded with granzyme B, immunolabeled with anti-granzyme and anti-rab4 antisera or anti-rab5 respectively. Rab4 positive endosomes gave red fluorescence and endosomes in which rab4 and granzyme B co-localize appeared yellow. A number of yellow endosomes can be seen suggesting that granzyme B is contained in an early endosome that is characterized by the marker rab4. As one would expect, there were also many rab4 positive endosomes that did not contain granzyme B which display a red colour (Figure 4.3). The observation that granzyme B label appeared in rab4 positive endosomes suggests that granzyme B was internalized via an endocytic process. Furthermore, the presence of granzyme B in rab4 negative endosomes that suggests that granzyme B may be transported to points further along the endosomal-lysosomal pathway (Stoorvogel et al., 1991).

The pattern of double label with rab4 and granzyme B is markedly different from that observed in double label of granzyme B-containing targets with anti-granyzme and anti-rab5. HeLa targets were incubated in soluble granzyme B for 60 minutes at 37°C, washed in PBS to clear the soluble granzyme. Cells were then fixed and immunolabeled with antiserum against granzyme and a monoclonal antibody against rab5 and the corresponding secondary antisera conjugated to Texas Red and FITC respectively. Figure 4.4 (Panel A) shows the rab5 labeling pattern given in green (FITC), Panel B shows the granzyme labeling in red (Texas Red) and Panel C shows the two fields combined. The green vesicles representing the rab5 endosomes are located at, or proximal to, the plasma membrane. The red vesicles representing sites of granzyme B accumulation are very small, but defined structures that are exclusive of

Figure 4.3 Internalized granzyme B co-localizes with the endosomal marker, rab4. Jurkat target cells were incubated for 60 minutes to allow uptake of soluble granzyme B. cells were attached to slides by centrifugation and fixed in 2% paraformaldehyde. Granzyme B and rab4 were visualized by indirect immunofluorescence with Texas Red- and FITC-conjugated secondary antibodies respectively. Panel (a) shows red label corresponding to rab4 localization. Panel (b) shows the field labeled with anti-granzyme B in green. Panel C is a combination of (a) and (b). Co-localization of granzyme B and rab4 is indicated by the appearance of yellow. Scale bar is 10 μm.

a
б
c

Figure 4.4 Double label of internalized granzyme B with the endosomal marker, rab5. HeLa target cells were incubated for 60 minutes to allow uptake of soluble granzyme B. cells were attached to slides by centrifugation and fixed in 2% paraformaldehyde. Granzyme B and rab5 were visualized by indirect immunofluorescence with Texas Red- and FITC-conjugated secondary antibodies respectively. Panel (a) shows red label corresponding to rab5 localization. Panel (b) shows the field labeled with anti-granzyme B in green. Panel C is a combination of (a) and (b). Sites of co-localization of granzyme B and rab5 near the plasma membrane are indicated by the appearance of yellow. The width of the field of view is 50 μm.

а
б
с

rab5 endosomes. It is possible, if not likely, that granzyme B passes through rab5 positive endosomes, but these figures demonstrate that vesicles defined by rab5 are not the site of granzyme accumulation.

Perforin/Ad2 functions to release granzyme B from endocytic vesicles

We have demonstrated that granzyme B can enter the target cell unassisted by perforin or Ad2, but the possibility remained that sufficient residual granzyme B was bound to the outside and that this could "piggyback" inside with perforin or Ad2 and result in death. To address this possibility, Jurkat cells were first allowed to internalize Ad2 and then treated with granzyme B. Since the rate of adenovirus internalization has a half-life of 10 minutes (Greber et al., 1993), 60 minutes was considered adequate to allow binding and internalization of the majority of Ad2. As an extra measure to ensure the total absence of virus on the external cell surface, the cells were washed under stringent conditions to remove bound virus (Greber et al., 1993) before the addition of granzyme B. Pre-treatment with Ad2, followed by granzyme B resulted in DNA fragmentation in target cells. This suggested that the granzyme B that was responsible for effecting the DNA fragmentation entered the target cell independent of Ad2. Furthermore, there was a persistence of the signal induced by Ad2 that was sufficient to stimulate trafficking of granzyme B inside the target cell.

Because we could not entirely exclude the possibility that the Jurkat cells contained residual membrane associated Ad2 at the time the granzyme B was added to the targets, a second approach was devised to test whether perforin or Ad2 could release granzyme B from intracellular vesicles. We took advantage of the fact that COS cells transfected with the granzyme B cDNA store the active enzyme in cytoplasmic vesicles. COS M5 cells expressing granzyme B were treated with perforin or Ad2 as described earlier for Jurkat and analyzed by fluorescent TUNEL assay in conjunction with immunolabeling with anti-granzyme antiserum to identify positively transfected

cells (Figure 4.5). Granzyme B positive cells exhibited rapid DNA fragmentation, while cells not expressing granzyme B were unaffected (Figure 4.5, panel D). Neither granzyme B activity nor protein has been detected in the supernatants or on the cell surface of transfected COS cultures (data not shown), therefore it appears that the granzyme is confined to intracellular vesicles. Thus, by two completely independent approaches we have separated the signals supplied by granzyme B and perforin/Ad2. Together these results suggest that both perforin and Ad2 affect the intracellular compartment occupied by granzyme B. In vivo granzyme B and perforin may be in the same vesicle but here they are likely internalized separately and granzyme B released after vesicle fusion.

DISCUSSION

As originally formulated, the granule exocytosis model of CTL mediated cytotoxicity envisaged lethal lytic effector molecules being delivered to target cells by vectoral exocytosis of CTL granules. Initially it was believed that perforin was sufficient to induce target cell lysis but then it became apparent that the granzymes (in particular granzyme B) were required to bring about physiologically relevant apoptotic death. The model was modified to include granzymes passing through perforin channels into the cytoplasm of the condemned cell. The formation of a complete macromolecular channel was not clearly demonstrated, but perforin damage to the membrane was a key factor in granzyme uptake. Despite the fact that many laboratories have been unable to replace perforin with a variety of channel forming and membraneolytic agents, this model has become the widely accepted paradigm.

Two signals are required for granzyme B-mediated death

Although there is a wealth of indirect evidence to suggest that granzyme B enters the target, this key experimental fact has not been established. We therefore

decided to look directly at whether granzyme B gains access to the target cell in the presence and absence of perforin. Using indirect immunocytochemistry and CLSM we found that granzyme B alone initially bound to the outer cytoplasmic membrane but then became internalized. In the absence of perforin, the granzyme remained cytoplasmic although it did appear to be concentrated around the nuclear membrane. Importantly we saw no evidence for the induction of apoptosis in these cells. In contrast when granzyme B and perforin were added together, we observed not only rapid uptake of the former but also clear evidence of target cell DNA fragmentation. Indeed target cells could also be preloaded with granzyme B and then induced to die by subsequent addition of perforin. Thus granzyme B alone can be taken up into a target cell but, in order for death to occur, perforin must also be present.

Endocytic internalization of granzyme B

In view of these results indicating that uptake of granzyme B is necessary but not sufficient for death, we were rather surprised by the observation that microinjection of the enzyme did cause target cell lysis. In a collaborative study, Dr. K. Tomaselli (IDUN Pharmaceuticals, La Jolla, CA) microinjected purified granzyme B into the cytoplasm of MCF-7 mammary carcinoma cells. These cells underwent apoptosis as measured by nuclear condensation with Hoescht 33342 stain. Clearly there must be some differences between the two systems for introduction of the granzyme. While microinjection delivers a protein to a defined location, it was unclear how cells exposed to soluble granzyme in the medium would achieve internalization. Previous experiments have shown that perforin can be replaced with a replication deficient strain of adenovirus, Ad2. We were able to show that Ad2 could also substitute for perforin in the experiments where target cells were pretreated with granzyme B. These observations provided a vital clue to the mechanism of granzyme entry.

The internalization and penetration of adenovirus is very well characterized (see Schenk, 1996). The virus is initially taken up by receptor mediated endocytosis into early endosomes. There is then a rearrangement of the outer penton base protein associated with a drop in endosomal pH that causes an increase in the hydrophobicity of the penton. This results in a disruption of the endosome and discharge of virus into the cytoplasm where it attaches to microtubules prior to translocation to the nucleus (Seth et al., 1986; Varga et al., 1991; Greber et al., 1993). The involvement of endocytosis and trafficking of Ad2 through endosomes led us to investigate the possibility that granzyme B follows a similar route.

The rab proteins are a family of small ras-like GTP binding proteins that serve a variety of functions at distinct points in the endocytic and secretory pathways (Chavrier et al., 1990; see also Novick and Brennwald, 1993; Gruenberg and Maxfield, 1995). Rab5 controls early endocytic events involving formation of the early endosome (Bucci et al., 1992) and rab4 appears to be involved in the sorting events involving recycling endosomes (van der Sluijs et al., 1992). There is some overlap in endosomes containing rab4 and rab5, but they are completely devoid of rab7, which is believed to be involved in further trafficking to lysosomes (Bottger et al., 1996). The distinct intracellular localization of these proteins make them particularly useful as markers of the protein trafficking pathways. Localization of granzyme B in a rab4 positive vesicle lends further evidence that entry and trafficking of granzyme B is through the endosomal pathway.

Compartmentalization in this manner provides a means to avoid continuation through the endosomal/lysosomal pathway which would result in degradation of the granzyme. This provides an explanation for the observation that target cells pulsed with granzyme B can be washed and rested for three hours prior to the addition of perforin without any negative effect on target cell apoptosis (Froelich, et al., 1996a). The presence of a mannose 6-phosphate moiety on granzyme B (Griffiths and Isaaz,

1993) could play a role in the trafficking of granzyme B within the target cell. Both the cation-dependent and -independent mannose 6-phosphate receptors are found in rab4 positive endosomes (Klumperman et al., 1993), and could inhibit or delay the progress of granzyme B though the endocytic pathway.

Release of granzyme B into the cytosol requires a membraneolytic event

Perforin and Ad2 have both have been shown to stimulate granzyme mediated apoptosis in targets loaded with granzyme B. Prior to the addition of the secondary stimulus, the granzyme is sequestered in endosomes where it appears to be innocuous to the target. After addition of perforin or Ad2 the apoptotic program is set in motion with the cleavage of granzyme B substrates and initiation of DNA fragmentation. The secondary signal clearly causes a re-localization of the granzyme to a site, or sites, where substrates are available. Ad2 is known to cause a disruption in endocytic vesicles (Seth et al., 1986, 1987; Seth, 1994) and perforin is also known to possess membrane disrupting properties (Masson and Tschopp, 1985). The observation that cells underwent apoptosis when treated first with Ad2 followed by addition of granzyme B, suggested that the signal stimulated by binding and/or entry of the virus persisted temporally to stimulate release of granzyme B. Although perforin was not required for endocytosis of granzyme B, it provided a critical second signal that appeared to result in the release of granzyme from its intracellular compartment. The delivery of granzyme into the cytoplasm may be mediated directly by insertion of perforin channels in endosomal membranes or by stimulating a signal that ultimately results in release of granzyme within the target cell. It is possible that a signal triggered by perforin may be entirely independent of its pore forming activity.

COS M5 cells transfected with granzyme B provided us with a system that allowed us to bypass the internalization process and address directly the issue of perforin or Ad2 triggering release of granzyme from an intracellular vesicle. Perforin

and Ad2 both induced rapid DNA fragmentation in granzyme B expressing COS cells and neither of these stimuli had adverse effects on mock transfected cells. Targeting of granzyme B to endosomes, or comparable vesicles, in COS cells could be via a mannose 6-phosphate receptor dependent process. This would resemble the intracellular trafficking of the proteinase cathepsin D where processing of the 55 kDa to the 44 kDa forms of the protein occurs in endosomes that are mannose-6-phosphate receptor positive (Rijnboutt et al., 1992). Thus, as the normal pathway targeting granzyme B to cytolytic granules of CTL (Peters et al., 1991; Griffiths and Isaaz, 1993) is not present in COS cells, the granzyme may remain in endosomes. It is unlikely that granzyme B is secreted by COS cells and re-internalized, since exhaustive efforts to detect granzyme B activity or protein in the supernatants of these cultures have been negative (data not shown).

Two signal model of CTL-mediated apoptosis

The studies presented in this chapter have been undertaken to dissect the killing pathway of cytotoxic T lymphocytes. We have shown that granzyme B can mediate its own uptake into the target and that perforin acts at a point downstream of entry. Contrary to the widely accepted model in which granzyme B internalization into target cells is facilitated by perforin channels, granzyme B is internalized by an endocytic process independent of perforin. Perforin activity, however, is absolutely required to provide the signal that results in the intracytoplasmic release from endosomes and subsequent nuclear translocation of granzyme B. Our results strongly imply that although the perforin channel mechanism does not appear to be operating at the plasma membrane perforin plays an intriguing role that is key to the apoptotic process, but until now, completely unexpected. In vivo it is likely that granzyme B and perforin are internalized in the same endosome. In our system, the granzyme B and the membraneolytic agent (perforin or Ad2) could be added separately, suggesting that

vesicles may fuse and thus release the granzyme. This could be relevant for the development of therapy based on the use of granzyme B as an inducer of apoptosis in pathogenic cells.

Once freed from the endosome the granzyme is able to cleave and activate substrates such as the CPP32-like caspases. It is clear, however, that this pathway is involved primarily in events involving DNA fragmentation. In contrast, granzyme B enzymatic activity is absolutely essential for setting in motion the whole apoptotic process. Novel substrates for granzyme B must exist that explain other features of apoptosis such as nuclear condensation and phosphatidylserine externalization. Finally, our results suggest that a search for other granzyme B-binding molecules, in addition to substrates, may reveal proteins that play an essential role in the internalization and intracellular transportation of this important cytotoxic effector.

CHAPTER V

TRAFFICKING OF GRANZYME B IN TARGET CELLS

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TRAFFICKING OF GRANZYME B IN TARGET CELLS

INTRODUCTION

Granzyme B has been shown to enter a target cell independently of other granule components (Pinkoski et al., submitted; Greenberg et al., 1997). We next undertook to better define the route followed by granzyme B at two stages: entry into the cell and subsequent intracellular trafficking. Determining the trafficking pathway and the control thereof will provide valuable insight into the regulation of granule mediated apoptosis.

Regulated uptake is important to eukaryotic cells because it provides a means to respond to environmental stimuli, to recover constitutively secreted proteins and as a defense against pathogens, toxins and other deleterious agents (for review see Mellman, 1996). Ironically, it is through the endogenous endocytic pathways that many toxins and pathogens gain access to the inside of a cell. Study of the cellular uptake and processing of pathogens and toxins has provided much of the current knowledge of endocytosis.

Cellular uptake of macromolecules may be facilitated by one of at least two routes of entry: receptor mediated endocytosis and fluid phase engulfment by pinocytosis (Mellman, 1996). Pinocytosis is a non-specific engulfment of the fluid phase by a cell, that involves rearrangement of the cytoskeleton and therefore can be inhibited by inhibitors of microtubule movement and other cytoskeletal dynamics. Proteins that gain access to a cell by pinocytosis are subsequently sequestered in cytoplasmic vesicles and are trafficked through the endosomal/lysosomal pathways.

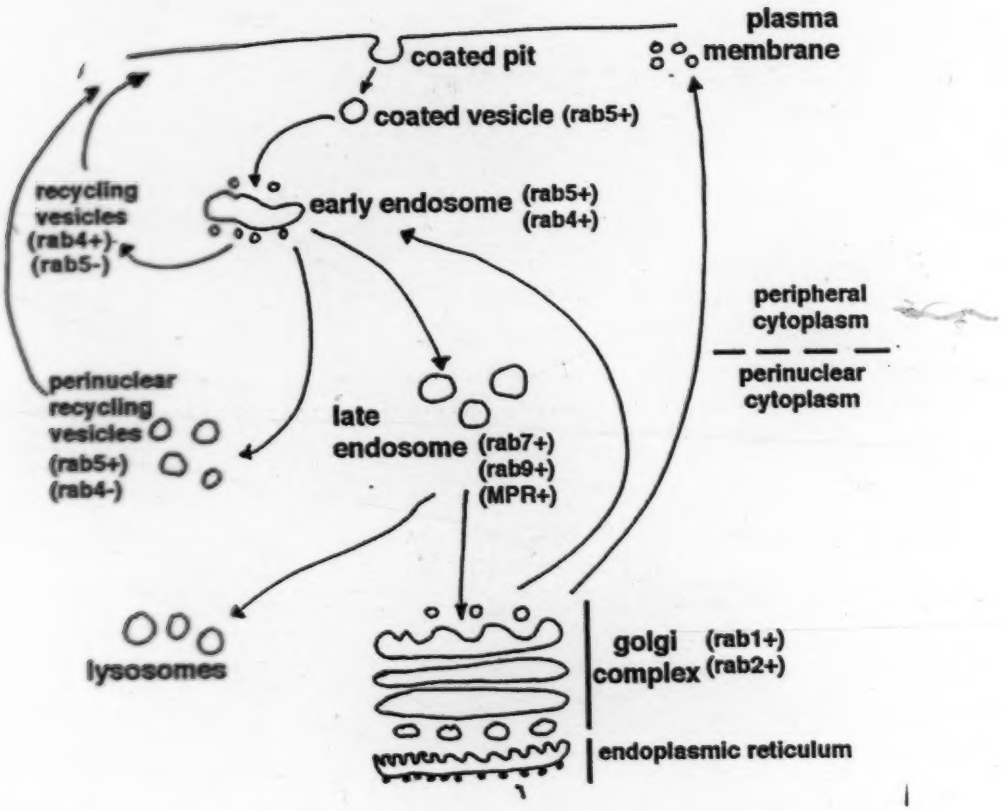
Conversely, receptor mediated endocytosis involves specific interaction between a membrane bound receptor and the ligand to be internalized (Figure 5.1) (Mellman, 1996). After a threshold of binding events is surpassed, receptor-ligand complexes aggregate and, usually in the presence of a clathrin or caveolin, form a coated pit. The coated pit invaginates within the plasma membrane and pinches off to form a new, membrane bound intracytoplasmic vesicle. This new vesicle, an early endosome, will mature by a series of membrane fusion events into a late endosome (Stoorvogel et al., 1991). Maturation of endosomes is accompanied by an acidification process that is driven by ATPase Na^+/H^+ or K^+/H^+ proton antiporter proteins. There is evidence to suggest that a second, pH-independent, pathway exists by which proteins are sorted that bypasses the late endosome to the lysosome step. This alternative pathway is not affected by the ATPase proton pump (van Weert et al., 1995).

Dissociation of ligand from receptor is believed to be facilitated by the drop in endosomal pH. The receptor is then recycled back to the plasma membrane to return to action and the ligand is subjected to further trafficking dependent on the nature of the ligand. Many ligands, having served the purpose of stimulating a response through the appropriate receptor, are trafficked to lysozomes and targeted for degradation.

After the maturation of the early to late endosome and dissociation of receptor from ligand, the receptor is recycled back to the plasma membrane for further duty and the ligand is sorted according to the appropriate pathway (Schmid et al., 1988). Sorting of receptors and ligands is controlled by the endosomal protein rab4. The transferrin receptor, another useful marker of the recycling vesicle, recycles back to the plasma membrane after internalization of transferrin and is co-localized with the rab4-positive endosome (van der Sluijs et al., 1992).

The mannose 6-phosphate receptor (MPR) is also involved in endocytosis and sorting (see Hille-Rehfeld, 1995). Glucuronidase is a lysosomal acid hydrolase that is transported to lysosomes by the MPR and is subsequently secreted and recovered by

Figure 5.1 Schematic diagram outlining basic trafficking during endocytosis. Ligand bound surface receptors aggregate in a coated pit. The coated pit invaginates and is pinched off to form a coated vesicle. Coated vesicles then mature into early endosomes where the sorting of ligands and receptors begins. Sorting through to late endosomes or into recycling vesicles is controlled by members of the rab family of proteins, namely rab4 and rab5.


plasma
membrane
coated pit
coated vesicle (rab5+)
recycling
vesicles
(rab4+)
(rab5-)
early endosome (rab5+)
(rab4+)
peripheral
cytoplasm
perinuclear
cytoplasm
perinuclear
recycling
vesicles
(rab5+)
(rab4-)
late
endosome (rab7+)
(rab9+)
(MPR+)
lysosomes
golgi (rab1+)
complex (rab2+)
endoplasmic reticulum

endocytosis (Gabel and Foster, 1986). Endocytosis and subsequent delivery to lysosomes is mediated through the MPR. Dissociation of M6P-bearing proteins and the MPR is believed to be driven by a pH-dependent mechanism that governs many of the processes in endosomal/lysosomal trafficking. In addition to uptake of cellular proteins, the MPR is also used to mediate entry of viruses and cellular toxins (Zhu et al., 1995a, 1995b).

Inhibitors of various steps in the endocytic and exocytic pathways have proved useful in determining the transport of proteins through established reticular networks. For example, brefeldin A fuses the membrane of the ER with the cis-golgi and prevents transfer of proteins between these two networks. Other useful trafficking inhibitors include chloroquine, monensin, bafilomycin A and nigericin which prevent the proper function of the ATPase Na^+/H^+ antiporter and thus inhibits endosomal/lysomal maturation.

Earlier we demonstrated that granzyme B can be taken up by a cell independently and Froelich et al. (1996) observed saturable binding sites for granzyme B on the surface of target cells. Taken together, these data suggest the existence of a receptor for granzyme B. To investigate the route of entry of granzyme B and to elucidate the requirements for entry we began with known physical characteristics of granzyme B that have been implicated in some form of cellular trafficking.

Granzyme B is glycosylated at two Asn residues (residues 51 and 162 of the mature protein) leading to a high mannose carbohydrate pattern. It has been demonstrated that trafficking of granzyme to cytolytic granules is mediated through interaction between mannose 6-phosphate residues on granzyme B and the mannose 6-phosphate receptor (MPR; Griffiths and Isaaz, 1993). It is not clear which of the two MPRs, the cation-dependent or cation-independent, is involved.

The earlier discovery that granzyme B is transported through an endosomal route led us to investigate the effects of trafficking inhibitors on granzyme B killing.

Since previous experiments describing the effects of trafficking inhibitors were performed with whole CTL and targets, it had been impossible to determine if inhibition of killing by such inhibitors as chloroquine and NH_4Cl were due to effects on the CTL or the target. In our CTL-free system, we can eliminate the effects on trafficking events in the CTL to elucidate the transport pathway of granzyme B in the target necessary to induce apoptosis.

Understanding the means by which granzyme B gains entry into the target cell and the intracellular trafficking pathway will provide us with valuable information toward our knowledge of cell mediated cytoxicity. We have discovered autonomous entry of granzyme, but we need to understand better how the target cell regulates granzyme movement and how the protease is ultimately brought in contact with substrates to trigger the induction of target cell apoptosis.

RESULTS

Entry of granzyme B is not mediated through the conserved KGD motif

One of the first clues to the means by which granzyme B binds to the surface of a target cell came with the observation that granzymes A, B, D, E, H and M all contain an Arg/Lys-Gly-Asp tripeptide sequence. The conserved motif is adjacent to the active site Ser residue in all granzymes except granzyme A, in which the KGD lies closer to the C-terminus. According to the molecular model of granzyme B (Murphy et al., 1987), the side chains of the KGD tripeptide, although in an internal portion of the proteinase, are accessible to the solvent and would be available for intermolecular interactions by virtue of their position near the binding pocket of the active site.

The (R/K)GD sequence is the means by which many viruses attach to integrin molecules on the surface of cells (Ruoslahti, 1996). Of the three residues the Arg may be replaced by a conservative substitution to Lys, but neither of the other two residues can bear even the most conservative of substitutions. KGD sequences are functionally

comparable to RGD, but neither RAD nor RGE possess binding affinity for integrins (Pierschbacher and Ruoslahti, 1984, 1987; Ruoslahti, 1996).

To test the possible involvement of KGD sequences in granzyme B entry into target cells, we used peptide inhibitors to attempt to block uptake of granzyme. This was carried out using a biological assay with the readout of cell death as a measure of granzyme uptake. Jurkat were treated for 60 minutes with one of the following inhibitory peptides GRGDSP or RGDS, or one of two negative control peptides, GRADSP and GRGES for 60 minutes followed by addition of granzyme B for 60 minutes and finally Ad2 for 60 minutes, all at 37°C. Virus treated cells were fixed, labeled by TUNEL and viewed by CLSM. None of the RGD inhibitory peptides, nor the negative control peptides adversely affected killing by granzyme B in this system.

Mannose moieties of granzyme B are not required for entry into targets but are required for the induction of apoptosis

Since transport to the secretory granule in CTL is mediated through interactions between mannose 6-phosphate on granzyme B and the mannose 6-phosphate receptor (MPR), we looked at the possible involvement of these moieties in trafficking of granzyme B in the target that leads to the induction of apoptosis. We undertook this line of investigation by studying the ability of an unglycosylated form of granzyme B to enter a target cell and cause death.

A granzyme B double mutant with the two glycosylated Asn residues converted to Ala (Asn51Ala and Asn162Ala) was generated by Dr. A. Caputo in Dr. Bleackley's laboratory. Using FPLC purified protein provided by Dr. Caputo, uptake and killing assays were performed with glycosylation deficient and wild type granzyme B. Jurkat target cells were incubated with approximately 1 μg/ml granzyme B at 37°C for 60 minutes. Cells were either fixed in paraformaldehyde for immuunocytochemical analysis or treated with Ad2 (10 pfu/cell) for an additional 60 minutes at 37°C. Cells

labeled with α-granzyme antiserum and viewed by confocal microscopy displayed an intracellular labeling pattern, showing that the unglycosylated form of granzyme B could enter the target cell.

The killing assay performed by adding Ad2 to cells pre-loaded with unglycosylated granzyme B yielded much different results from those treated with wild type granzyme B. Cells treated with unglycosylated granzyme B did not display DNA fragmentation, PS externalization, DNA hypoploidy (severe DNA degradation) or cell shrinkage (Figure 5.2).

An additional approach to investigate the requirement of glycosyl moieties for granzyme B killing utilized the COS cell system. Granzyme B and the granzyme B glycosylation mutant were transiently expressed in COS M5 cells and incubated with Ad2. Cells were labeled with anti-granzyme antiserum and TUNEL assessed by CLSM . The labeling patterns of both granzyme B variants were similar to those observed in the absence of Ad2 and only the wild type granzyme B induced DNA fragmentation.

Mannose 6-phosphate partially inhibits killing by granzyme B

Glycosylation appears to be important for the proper delivery of granzyme B within a target cell. To further characterize the requirement of granzyme B glycosyl moieties for biological activity, we performed competition experiments with soluble mannose and mannose 6-phosphate (M6P). Jurkat targets were pre-treated with soluble mannose 6-phosphate or mannose (5 mM) prior to treatment with granzyme B (1μg/ml) and Ad2 (10 pfu/cell). Targets were labeled by TUNEL, annexin V-FITC or PI and assayed by flow cytometry. Figure 5.3 summarizes the effects of treatment with these two sugars. Treatment of targets with M6P resulted in a partial inhibition of killing, whereas unphosphorylated mannose had no inhibitory effect. This suggests that granzyme B trafficking may be mediated, in part, by the MPR.

Figure 5.2 Effects of glycosylation state of granzyme B on killing. Jurkat target were treated with soluble granzyme B, wild type enzyme and a double mutant that is unable to be N-linked glycosylated, and Ad2. Apoptosis was measured by FACS analysis of cells labeled with annexin V-FITC, TUNEL and propidium iodide. Cell volume was assessed by forward scatter in FACS analyses. Both forms of granzyme B were tested for enzymatic activity. The unglycosylated form of granzyme B was enzymatically active but did not demonstrate any biological activity. Specific killing was calculated by [killing - spontaneous death] / [killing by granzyme B and Ad2 - spontaneous death] x 100. Killing by granzyme B and Ad2 in the absence of additional compounds was set at 100%.

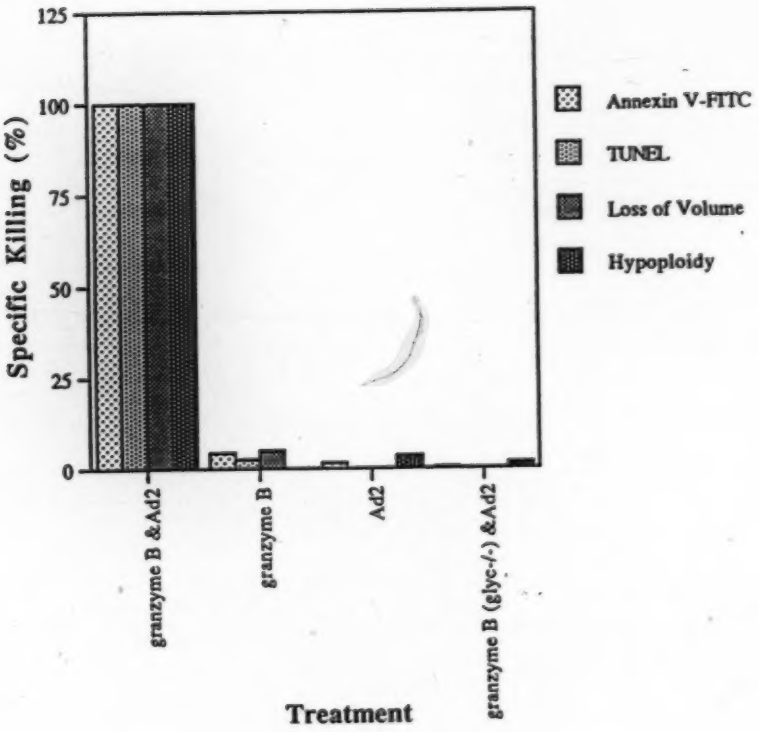
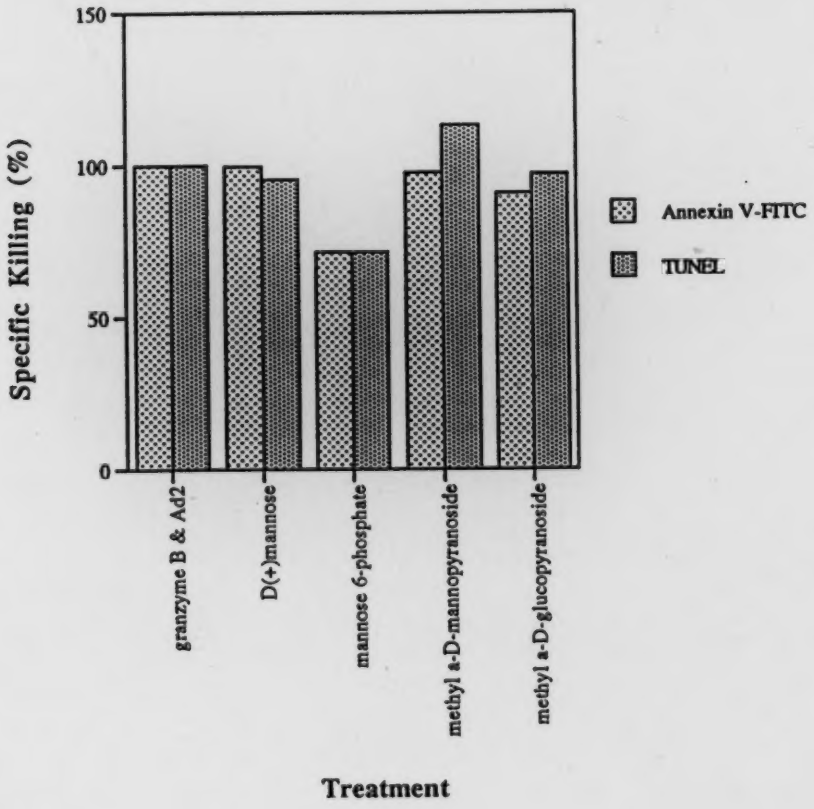

125
100
75
50
25
0
Specific Killing (%)
Annexin V-FITC
TUNEL
Loss of Volume
Hypoploidy
granzyme B &Ad2
granzyme B
Ad2
granzyme B (glyc-/-) &Ad2
Treatment

Figure 5.3 Contribution of mannose 6-phosphate to granzyme B mediated killing. Jurkat targets were treated with mannose, mannose 6-phosphate, methylmanno-pyrannoside and methylglucopyrannoside (each at 5 mM) for 30 minutes at 37°C, prior to treatment with granzyme and Ad2 as previously described The sugars were present throughout the entirety of the experiments. Cell death was measured by FACS analysis of annexin V-FITC and TUNEL labeled cells. Specific killing was calculated by [killing - spontaneous death] / [killing by granzyme B and Ad2 - spontaneous death] x 100. Killing by granzyme B and Ad2 in the absence of additional compounds was set at 100%.


150
100
50
0
Specific Killing (%)
granzyme B & Ad2
D(+)mannose
mannose 6-phosphate
methyl a-D-mannopyranoside
methyl a-D-glucopyranoside
Treatment
Annexin V-FITC
TUNEL

Entry appears to be via receptor mediated endocytosis

To test the possibility that granzyme B was entering the target cell by fluid phase engulfment, we performed a series of temperature block experiments. At 4°C pinocytosis and endocytosis are inhibited due to the rigidity of the plasma membrane (Mellman, 1996). By incubating targets in granzyme B at 4°C for 60 minutes we prevent the uptake of granzyme by fluid phase engulfment. The cells were washed to remove any unbound granzyme B and then incubated in Ad2 at 37°C for an additional 60 minutes. Death was measured by annexin V-FITC binding and TUNEL and analyzed by FACS.

In this biological assay, death should occur only if sufficient granzyme B remains bound to the cell surface through the washing process. In these experiments we found that incubation of targets in granzyme at 4°C followed by washing and addition of virus at 37°C showed no significant decrease in the level of killing as measured by TUNEL and PS externalization (Figure 5.4).

Killing by granzyme B is inhibited at 20°C

Another useful temperature block used to study intracellular transport is incubation at 20°C. At 20°C cells are still able to take up molecules, but the transport of proteins from early to late endosomes and from early endosomes to the trans-golgi network is inhibited (Galloway et al., 1983; Dunn et al., 1989). Incubation of targets with granzyme B at 20°C did not inhibit uptake of granzyme B, as seen by immunocytochemical staining and visualization by CLSM (data not shown). However, subsequent treatment of granzyme loaded targets with Ad2 or perforin at 20°C did not result in DNA fragmentation or PS externalization (Figure 5.5). A similar result was obtained when the targets were treated with granzyme B at 37°C for 60 minutes and then shifted to 20°C for an additional 60 minutes upon addition of Ad2 or perforin.

Figure 5.4 Use of the 4°C temperature block suggests that entry of granzyme B is mediated by a receptor mediated mechanism. Jurkat targets were treated with soluble granzyme B at 4°C for 60 minutes. The cells were washed in PBS at neutral pH or in acid-citrate buffer (pH 3.0) and treated with Ad2 for 60 minutes at 37°C. Non-specific engulfment of the fluid phase is inhibited at 4°C. Acid wash is used to remove particles bound to the external surface of the plasma membrane. Specific killing, as assayed by annexin V-FITC and TUNEL, is measured relative to death observed by treatment with granzyme B at 37°C, washed at neutral pH, followed by treatment with Ad2 at 37°C for 60 minutes.

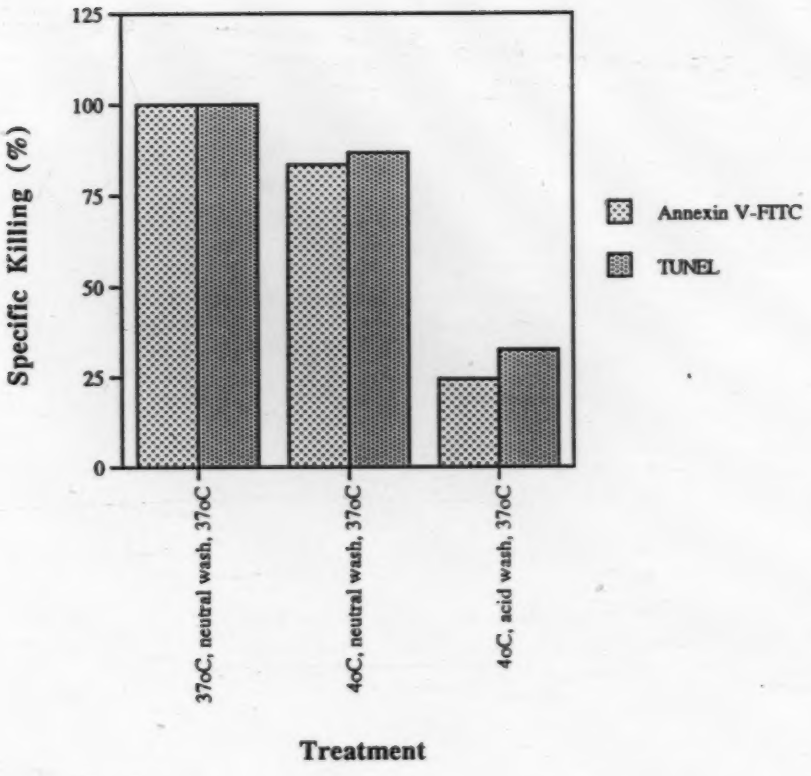
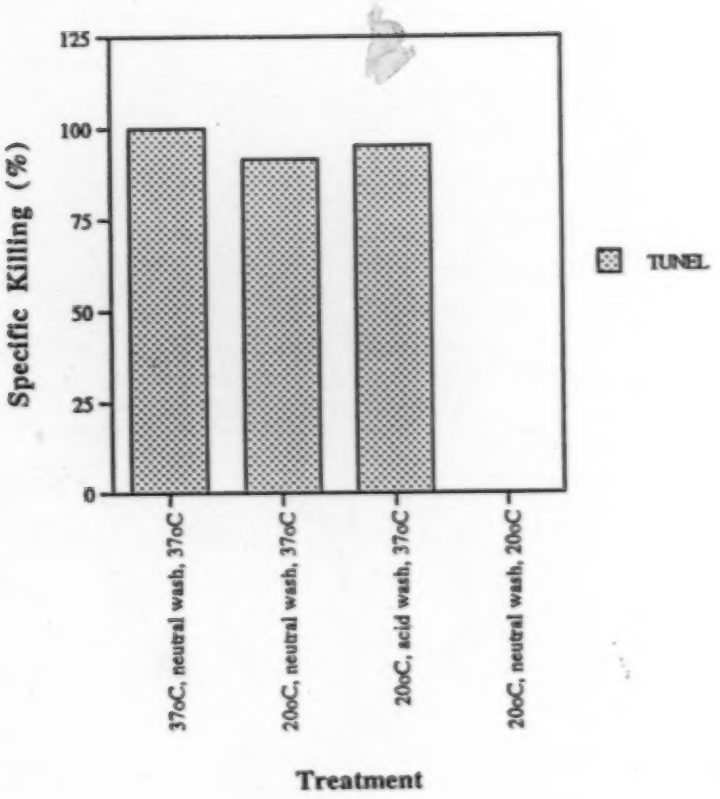

125
100
75
50
25
0
Specific Killing (%)
Annexin V-FITC
TUNEL
37oC, neutral wash, 37oC
4oC, neutral wash, 37oC
4oC, acid wash, 37oC
Treatment

Figure 5.5 Internalization of granzyme B is not inhibited by incubation at 20°C. Jurkat targets were incubated in soluble granzyme B for 60 minutes at 20°C, followed by neutral or acid wash and treatment with Ad2. Cell death was measured FACS analysis of TUNEL labeled cells. Specific killing was calculated by [killing - spontaneous death] / [killing by granzyme B and Ad2 - spontaneous death] x 100. Acid wash of cells treated at 20°C did not adversely affect killing suggesting that granzyme B had been internalized, but there is a marked inhibition of killing by continued incubation at 20°C


125
100
75
50
25
0
Specific Killing (%)
TUNEL
37oC, neutral wash, 37oC
20oC, neutral wash, 37oC
20oC, acid wash, 37oC
20oC, neutral wash, 20oC
Treatment

Inhibition of killing by agents that disrupt pH balance

Maturation of early endosomes into late endosomes and further into lysosomes is accompanied by a sequential acidification of vesicles (pH 6.8 to 5.2; Mellman, 1996). There are a number of useful inhibitors employed for the study of endocytosis and subsequent trafficking. Inhibitors of the vacuolar ATPase proton pump disrupt the ability to maintain the pH gradient in many endosomal and lysosomal vesicles and have been shown to inhibit CTL mediated killing (Ausiello et al., 1984). However, it could not be determined which cell, the CTL or target, was being affected by these agents. Some of these inhibitors include NH_4Cl (Ausiello et al., 1984), chloroquine (Pelassy and Aussel, 1993; Bi et al., 1996), nigericin (Pless and Wellner, 1996), bafilomycin A (Bowman et al., 1988; Umata et al., 1990; Yoshimori et al., 1991), concanamycin A (Kataoka et al., 1996) and monensin (Mollenhauer et al., 1990). Brefeldin A is another useful trafficking inhibitor that causes a fusion of the cis-golgi with the ER is useful to inhibit transport between these two structures (Fujiwara et al., 1988).

To test the dependence of granzyme B trafficking on pH changes we tested a number of inhibitors of the ATPase Na^+/H^+ proton antiporter. Jurkat targets were pretreated with a number of potential inhibitors for 60 minutes prior to treatment with granzyme B and Ad2 as described above. Chloroquine and nigericin had no appreciable affect on granzyme B-mediated killing and bafilomycin, monensin and NH_4Cl showed partial inhibition of killing. These data are summarized in Figure 5.6. Brefeldin A had no inhibitory effect on granzyme B induced death (not shown).

The trafficking inhibitor experiments were repeated in whole CTL killing assays to compare the effects on killing by CTL versus killing by soluble granzyme B. A granzyme B expressing CTL line, wt, was incubated with L1210 targets in the presence of bafilomycin, chloroquine, NH_4Cl, and nigericin. Death was measured by ^{51}Cr-release and ^3H-thymidine release assays. The results are summarized in Figure 5.7.

Figure 5.6 Inhibition of granzyme B/Ad2 killing by inhibitors of the ATPase Na^+/H^+ antiporter. Jurkat targets were treated with the indicated trafficking inhibitor for 60 minutes at 37°C prior to the addition of granzyme B and Ad2. Cell death was measured by FACS analysis of annexin V-FITC and TUNEL labeled cells. Specific killing was calculated by [killing - spontaneous death] / [killing by granzyme B and Ad2 - spontaneous death] x 100.

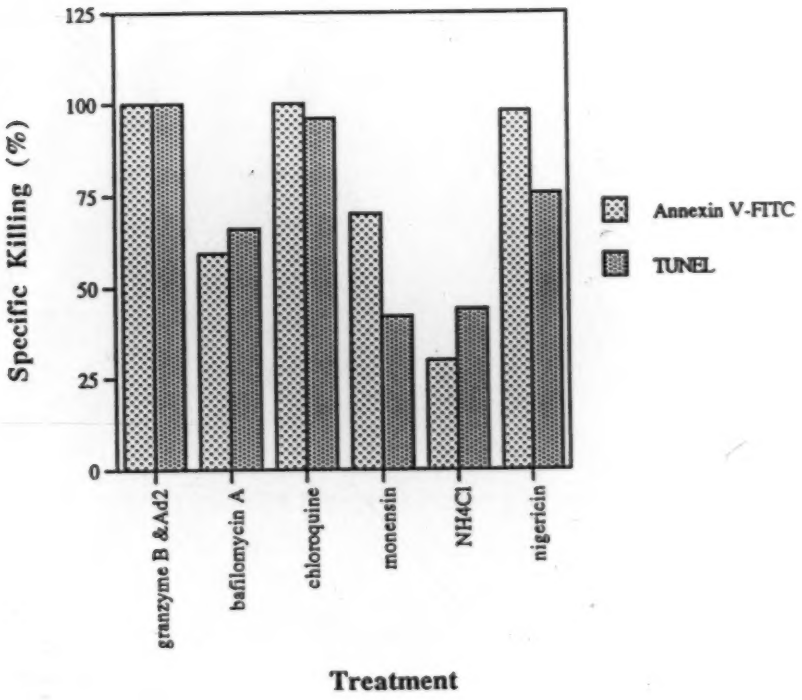
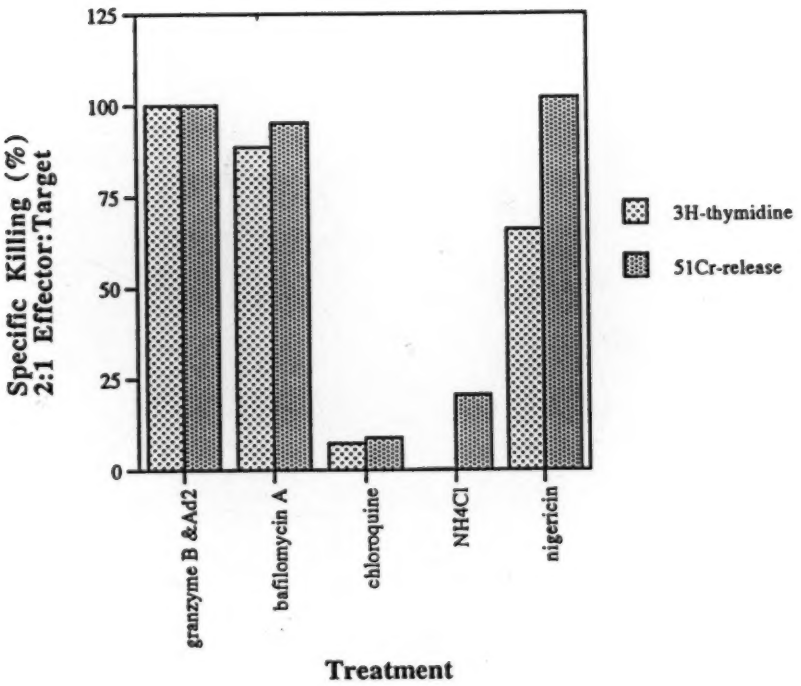

125
100
75
50
25
0
Specific Killing (%)
granzyme B &Ad2
bafilomycin A
chloroquine
monensin
NH4Cl
nigericin
Treatment
Annexin V-FITC
TUNEL

Figure 5.7 *In vivo* inhibition of granzyme B/Ad2 killing by inhibitors of the ATPase Na^+/H^+ antiporter. Radiolabeled L1210 targets were treated with the indicated trafficking inhibitor for 60 minutes at 37°C prior to the addition of CTL. Cell death was measured by ^{3}H-thymidine- and ^{51}Cr-release assays. Specific killing was calculated by [killing - spontaneous death] / [killing by granzyme B and Ad2 - spontaneous death] x 100.


Specific Killing (%)
2:1 Effector:Target
125
100
75
50
25
0
granzyme B &Ad2
bafilomycin A
chloroquine
NH4Cl
nigericin
Treatment
3H-thymidine
51Cr-release

Chloroquine and NH_4Cl both inhibited killing, but only NH_4Cl had any inhibitory effect on killing in the CTL-free system (Figure 5.6). Thus, it appears that inhibition by chloroquine is occurring in the CTL, whereas inhibition by NH_4Cl is effecting events in both the CTL and the target.

DISCUSSION

Entry of granzyme B is not mediated through the RGD/KGD motif

Ad2 binds to cell surface integrins via the RGD integrin recognition sequence. When we started looking for a possible mode of entry for granzyme B, we looked for clues contained in the primary protein sequence. We found that six granzymes contain RGD or KGD sequences at or near the active site. The possibility of granzyme entry mediated by KGD motifs was attractive since this tripeptide is conserved in six granzymes. KGD and RGD sequences are utilized by a number of viruses to interact with integrins and we believed that this may be an example of viral strategies recapitulating mechanisms of the host immune system. However, a number of short RGD-containing inhibitory peptides had no deleterious effect on granzyme B entry or killing. This ruled out one of the possible direct modes of entry but did not rule out the possibility of receptor mediated endocytosis of granzyme B.

The mannose phosphate receptor in cell mediated cytotoxicity

A role for MPR in cell mediated cytotoxicity was proposed in 1981 when Forbes et al. demonstrated that mannose 6-phosphate, added to a cytotoxicity assay, could effectively inhibit NK cell killing of targets. This result was refuted in 1985 when Haubeck et al. showed that NK cells from patients with I-cell disease, where granzyme B acquires complex glycans instead of high mannose glycosylation patterns, did not display any reduction in NK activity, nor was there any inhibition by M6P. However, since Griffiths and Isaaz (1993) proposed the existence of a parallel pathway

for regulated secretion of granule constituents in patients with I-cell disease, there remains the possibility that MPR does in fact play a role in cell mediated cytotoxicity. Such a compensatory pathway in patients with disease may account for the lack of reduction of NK activity.

In addition to controlling intracellular trafficking, the MPR is also known to mediate uptake of M6P containing proteins (Hille-Rehfeld, 1995). However, in competition studies with M6P there was no significant decrease in the number of cells taking up granzyme B. Since this assessment was made by visual microscopic analysis, it is possible that there may have been a reduction in the amount of granzyme taken up by each positive cell.

Although M6P did not appear to be adversely affecting entry of granzyme B, it did inhibit killing by 25%. This result appears to be significant since unphosphorylated mannose did not have any inhibitory effect on killing. Carbohydrate moieties on granzyme play some critical role in target cell trafficking of granzyme B leading to death, since the deglycosylated form of granzyme B failed to induce any aspects of apoptotic death assessed. Perhaps there is some contribution from the core carbohydrate that is partially dependent on M6P and independent of mannose.

Acidification may contribute to trafficking of granzyme B

From our earlier data we have shown that granzyme B co-localized with an early endosomal marker. Transport through the trans-golgi network to the endoplasmic reticulum is not likely since there was no inhibition of killing with brefeldin A, but there may be a contribution of acidification to trafficking through endosomal vesicles. However, only partial inhibition of killing was observed with bafilomycin, monensin and NH_4Cl and no inhibition was observed in the presence of chloroquine and nigericin. This was a rather perplexing observation since the infective process of Ad2 involves pH-dependent conformational changes of the penton base protein. Changes in

the penton base facilitate penetration from endosomes into the cytosol (Varga et al., 1991).

A lack of inhibition by agents that disrupt acidification led to two important conclusions: trafficking of granzyme B in target cells is not entirely pH-dependent and the perforin-like effect of the Ad2 that triggers the initiation of granzyme mediated apoptosis is independent of, or precedes, the penetration of virus into the cytosol. It will be necessary to test the sensitivity of Jurkat targets to these agents. It is possible that a lack of sensitivity may be due to cellular resistance rather than pH-independent trafficking of granzyme B.

Temperature dependence of granzyme B mediated killing

Froelich et al. (1996) used radiolabeled granzyme B to demonstrate the presence of measurable and saturable binding sites for granzyme B on the surface of Jurkat targets. Our data from the 4°C pre-treatment of cells with granzyme followed by release at 37°C is consistent with Froelich's observations. Interaction between granzyme B and a surface receptor must be of sufficiently high affinity to occur at 4°C and withstand the washing procedure prior to addition of perforin or Ad2 and incubation at 37°C.

Although incubation at 20°C did not inhibit internalization of Ad2, it is possible that penetration may be compromised. However, if this is the case, then results would demonstrate that the Ad2-dependent activity that results in the release of granzyme was inhibited by incubation at 20°C. That is, the membraneolytic events induced by Ad2 is downstream of virus internalization.

These data are consistent with some of the earliest observations of cytotoxic cell killing. Zagury et al. (1975) demonstrated that peritoneal exudate lymphocytes form conjugates with EL4 targets efficiently at 22°C, but there is no killing at this temperature (Berke et al., 1972; Zagury, 1975). The early studies could not determine

if the nature of the inhibition of killing at 22°C, but here we clearly demonstrated an inhibition of events occurring within the target cell. These data do not rule out the possibility of concurrent inhibition of killer cells at 22°C. The CTL used in at least one of these studies was later determined to be killing via the Fas pathway. However, there remains the possibility that the 20°C block in killing may represent a common mechanism of inhibition for both granzyme and Fas pathways or may represent a block at a common point after the two pathways have merged.

There remains the possibility that there is more than one means of entry, or that there is more than one contributing factor for efficient entry. In this way, mannose 6-phosphate would contribute to uptake, but would not be wholly responsible. To address this possibility inhibition studies will have to performed with permutations of known and suspected inhibitors to assess the contribution of each.

Although the details of granzyme B trafficking in the target cell remain to be elucidated, it is now clear that granzyme B is subjected to regulated transport in the target cell. The existence of a granzyme B receptor opens exciting possibilities for selective sensitization to killing by granzyme B. Identification of a surface granzyme receptor and the elucidation of the trafficking requirements for granzyme B will provide us with valuable information with potential therapeutic applications in chemotherapy.

We have demonstrated that granzyme B is subjected to trafficking by the target cell. Transport of granzyme B contributes to the killing, since inhibition of granzyme trafficking inhibits apoptosis. Although the path followed by granzyme B inside the target requires greater refinement, we have shown that trafficking is dependent on the glycosylation state of the enzyme and mediated in part by the mannose 6-phosphate receptor. Dependence on acidification of endosomes is unclear but remains a possibility.

CHAPTER VI

NUCLEAR EVENTS IN GRANZYME B-MEDIATED KILLING: NUCLEAR TRANSLOCATION OF GRANZYME B COINCIDES WITH CELL DEATH

CHAPTER VI

NUCLEAR EVENTS IN GRANZYME B-MEDIATED KILLING: NUCLEAR TRANSLOCATION OF GRANZYME B COINCIDES WITH CELL DEATH[1]

INTRODUCTION

Disintegration of the nucleus is an integral step in apoptosis. Nuclear condensation and chromatin margination were observed early in the study of apoptosis. These morphological observations were followed by the biochemical description of nuclear breakdown, which involves nucleolytic breakdown of DNA into multimers of internucleosomal sized fragments (approximately 180 bp). The "laddering" effect described by Duke et al. (1983) has become one of the signature features of apoptosis.

The regulation of DNA fragmentation has remained an elusive mystery for some time, but it is known that chromosomal degradation occurs in two distinct steps: DNA breakdown to 50-300 kb fragments (Oberhammer et al., 1993), followed by fragmentation into nucleosomal ladders (Duke et al., 1983). Until recently, researchers have only been successful in determining the conditions under which the nuclease(s) are active. A dependence on divalent cations, Ca^{2+} and Mg^{2+}, appears to be the most common feature of the apoptotic nuclease(s). It is not known if the Ca^{2+} / Mg^{2+} dependence is a direct requirement or a dependence of some upstream nuclease activating activity.

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[1] A version of this chapter has been submitted for publication to the Journal of Cell Biology.

Nuclear condensation appears to occur through proteolytic breakdown of the nuclear lamina. Lamins A, B and C provide the framework on which the nuclear envelope is supported (McKeon, 1991). Lazebnik et al. (1995) and Takahashi et al. (1996) have shown that cleavage of lamin A can be attributed to caspase-6 (Mch2α).

The caspases were implicated in nuclear apoptotic events when it was shown that caspase-3 cleaved PARP (Nicholson et al., 1995). Activation of caspase-3 by granzyme B is necessary to induce DNA fragmentation in granule mediated killing by CTL (Darmon et al., 1996). Caspase-3 has recently been shown to be involved in another aspect of DNA fragmentation. DFF is cleaved and activated by caspase-3. Once activated DFF is responsible for the activation of an endonuclease activity that gives rise to nucleosomal laddering. Therefore, by cleavage of DFF and PARP, caspase-3 initiates DNA fragmentation and upsets the repair activity to compensate for the damage.

Transport of proteins into the nucleus appears to be mediated by one of two known mechanisms: passage through the nuclear pore complex (NPC) or by passive diffusion (see Davis, 1995; Nigg, 1997). Entry through the NPC requires the presence of a nuclear localization signal (NLS). The most commonly observed NLS are continuous stretches of lysines and arginine residues. There are also bipartite NLS that also have stretches of lysine and arginine residues but may be discontinuous. It has been demonstrated that the NLS from such proteins as SV40 large T antigen (linear: KKKRK) or nucleoplasmin (bipartite: KR-x_{10}-KKKK) are necessary and sufficient for nuclear translocation of SV40 T antigen and nucleoplasmin respectively and that each is sufficient to direct nuclear transport of chimeric BSA containing the NLS. In addition to direct transport through the NPC via NLS, there are also shuttle proteins that interact with proteins to carry them into the nucleus

Granzyme B is known to participate indirectly in nuclear apoptotic events, such as DNA fragmentation through the activation of caspase-3 and nuclear condensation

through the activation of caspase-6. There is a growing body of evidence suggesting a direct nuclear activity for granzyme B (Pinkoski et al., 1996; Trapani et al., 1996). Granzyme B binds to a nucleolar protein associated with heterochromatin *in situ* and now granzyme B has been shown to undergo rapid nuclear translocation in target cells that is associated with DNA fragmentation. The studies presented in this chapter outline the contribution of granzyme B to such nuclear events as DNA fragmentation, nuclear condensation and lamin proteolysis. Since the preferred cleavage site for granzyme B was determined (D.W. Nicholson, personal communication), it appeared that lamin A may be a substrate for granzyme B. The possibility that granzyme B cleaves lamin A seemed a reasonable line of investigation since granzyme B has been shown to activate caspases and thus can enter the death pathway by substituting for caspase activities.

RESULTS

Cell death is concomitant with nuclear translocation of granzyme B

It has been proposed that granzyme B, in addition to cleaving cytosolic substrates, contributes to apoptosis through a mechanism that involves entering the nucleus (Froelich et al., 1996b; Pinkoski et al., 1996; Trapani et al., 1996). Therefore, we asked if, after cells have been exposed to granzyme B, treatment with perforin or Ad2 resulted in translocation of granzyme B to the nucleus. Jurkat and YAC-1 target cells were incubated with granzyme B and then exposed to sublytic doses of perforin or Ad2. Granzyme B was detected by immunolabeling and apoptosis assessed by fluorescence TUNEL or DAPI stain and viewed by CLSM or fluorescence microscopy, respectively. Figure 6.1 shows typical TUNEL positive Jurkat cells after treatment with granzyme B and perforin or Ad2 (panels a and b). Granzyme B was no longer observed in a distinct punctate cytoplasmic pattern, but rather, significant nuclear labeling of granzyme B was observed associated with regions of TUNEL label.

Figure 6.1 Nuclear translocation of granzyme B is associated with DNA fragmentation. Jurkat targets were incubated for 60 minutes to allow uptake of granzyme B. After washing, cells were treated with perforin (a), Ad2 (b), and anti-Fas antibody (c). Cells in all panels were labeled with anti-granzyme B antiserum and Texas Red-conjugated goat anti-rabbit antibody (red) and by TUNEL with dUTP-FITC (green). Regions of co-localization appear yellow. Scale bar is 10 μm.

a
б
c

The NIH-Image software was used to analyze digital confocal micrographs to quantitate relative cytoplasmic and nuclear levels of granzyme B. Black and white micrographs were sharpened, edged and analyzed by counting grains of positive pixels with signals above a pre-set threshold. A summary of micrographs analyzed in this manner in given in Tables 6.1 and 6.2. Jurkat cells pre-treated with granzyme B had less than 10% of the total granzyme B label in the nucleus after 2 hours (Table 6.1). This was considered to represent background for quantitation by this method since it is comparable to the level of nuclear granzyme B observed in transfected COS cells (Table 6.2). After treatment with perforin, up to 57% of the total granzyme B was nuclear. Ad2 infection of granzyme B treated cells resulted in even greater levels of nuclear granzyme, up to 99%. Interestingly, detection of granzyme B in the nuclei of target cells preceded DNA fragmentation and cell death. Cells pre-treated with granzyme B and washed prior to Ad2 infection showed similar levels of nuclear granzyme as compared to cells treated concurrently with granzyme and Ad2. Jurkat treated first with Ad2 before addition of soluble granzyme B also contained significant levels of the proteinase in the nucleus, up to 75% of the total granzyme.

Enzymatic activity of granzyme B is required for apoptosis, but not for entry into the target cell

We next asked whether catalytically active granzyme B was necessary for endocytosis, nuclear translocation and DNA fragmentation. In the first approach, the cellular distribution of inactive granzyme was determined. Granzyme B was chemically inactivated with the serine protease inhibitor 3,4-dichloroisocoumarin (DCI), which is known to inhibit granzyme B activity (Hudig et al., 1991), and then added to target cells in the presence and absence of Ad2. Cells were immunolabeled for detection of granzyme B and analyzed by CLSM. The distribution of granzyme B in targets treated only with the inactive protease was limited to the cytoplasm. However, following

treatment with Ad2, the inactive granzyme underwent translocation to the nucleus. As shown in Table 6.1, prior inactivation of granzyme B with DCI did not adversely affect the increase in nuclear granzyme signal, with nearly half of the total granzyme B localized in the nucleus within 60 minutes following Ad2. Importantly, the target cells did not display evidence of DNA fragmentation by TUNEL or annexin V binding. Similarly, treatment of cells with purified granzyme B[S183A] did not undergo apoptosis, but there was a significant accumulation of intracellular granzyme. Nuclear granzyme B[S183A] localization was stimulated following addition of Ad2 or perforin, albeit at reduced levels (data not shown).

In a second approach the trafficking of the granzyme B was evaluated in COS cells that expressed either active proteinase or an active site mutant (Caputo et al., 1994). After Ad2 stimulation of transfected COS cells, granzyme B positive cells displayed a high level of nuclear granzyme B and were essentially 100% TUNEL positive (Figure 6.2). COS cells expressing the inactive S183A mutant form of granzyme B also displayed Ad2 dependent nuclear labeling of granzyme B, but were not TUNEL positive (Figure 6.2). Analysis of CLSM micrographs showed there was a five-fold increase of granzyme B in nuclei of cells treated with granzyme B[S183A] active site mutant (Table 6.2). It is important to note the increase in relative nuclear inactive granzyme B in the absence of DNA fragmentation and TUNEL reactivity, however the level of nuclear granzyme B[S183A] was lower than wild type granzyme B. Results from these two approaches demonstrate that endocytic uptake and nuclear translocation of granzyme B is not dependent on enzymatic activity. However, the use of inactivated or mutant granzyme B resulted in the complete absence of the biochemical and morphological indicators of apoptosis.

Figure 6.2 Apoptosis, but not nuclear translocation of granzyme, of COS expressing granzyme B is dependent on the presence of an active enzyme. COS M5 were transfected with granzyme B (panels a and b), or granzyme B[S183A] (panel c) followed by buffer (panel a) Ad2 (panels b and c). Cells in all panels were labeled with anti-granzyme B antiserum and Texas Red-conjugated goat anti-rabbit antibody (red) and by TUNEL with dUTP-FITC (green). Regions of co-localization appear yellow. Scale bar is 10 μm.

a
б
c

Treatment 1		Treatment 2		% TUNEL positive	% nuclear granzyme B
granzyme B	60 minutes			0	7.86
	120 minutes			0	9.63
Ad2	2 hours			<1	0.00
granzyme B & Ad2					
	30 minutes			5-10	95.07
	60 minutes			>25	85.81
	2 hours			>50	
	4 hours			<5	
granzyme B	60 minutes	Ad2	1 hour	>10	99.36
granzyme B	60 minutes	Ad2	2 hours	15-25	97.31
granzyme B	60 minutes	Ad2	4 hours	60	85.77
granzyme B	60 minutes	perforin	30 minutes	5-10	57.02
granzyme B	60 minutes	perforin	60 minutes	60-65	46.58
granzyme B	60 minutes	perforin	2.5 hours	not determined	55.25
granzyme B/DCI					
	60 minutes			0	49.41
DCI	60 minutes			0	not applicable

Table 6.1 Nuclear accumulation of granzyme B in Jurkat target cells. Cells were treated sequentially with soluble granzyme B at 1μg/ml and Ad2 (10 pfu / cell) or perforin (90 H.U./ ml). Quantitation of total cellular and nuclear granzyme B label was performed on digitally acquired confocal micrographs using the NIH-Image program.

Treatment 1	Treatment 2	% TUNEL positive	% nuclear granzyme B
granzyme B 60 minutes		0	6.02
granzyme B 60 minutes	Ad2 1 hour	35-40*	84.5
granzyme B 60 minutes	Ad2 2 hours	<15	not determined
granzyme B 60 minutes	perforin 2 hours	>50	71.92
granzyme B DEVD	Ad2 1 hour	<1	17.81
S183A active site mutant	Ad2 1 hour	<0.1	29.82
S183A active site mutant	perforin 1 hour	0	not determined

* 100% of granzyme B positive cells were TUNEL positive

Table 6.2 Nuclear accumulation of granzyme B in COS M5 cells transfected to express granzyme B or granzyme B[S183A] active site mutant. After a 48 hour transfection, cells were treated with Ad2 (10 pfu / cell) or perforin (90 H.U./ ml). Quantitation of total cellular and nuclear granzyme B label was performed on digitally acquired confocal micrographs using the NIH-Image program.

Caspase-3 is involved in granzyme B-mediated DNA fragmentation, but not nuclear condensation

Granzyme B has been shown to cleave and activate caspase-3 in vitro (Darmon et al., 1995) and to be processed in targets exposed to either whole CTL or the combination of granzyme B and Ad2 (Darmon et al., 1995; Froelich et al., 1996). To investigate the role of caspase-3 in the development of apoptotic morphology, the effect of the caspase-3 inhibitor, z-DEVD-fluoromethylketone (z-DEVD-fmk) was evaluated in our model system. In the presence of z-DEVD-fmk, Jurkat and COS M5 targets treated with granzyme B and Ad2 failed to develop TUNEL reactivity (Figure 6.3), suggesting that the inhibitor prevented the form of DNA fragmentation recognized by this technique. Despite the absence of DNA fragmentation, immunolocalization studies showed that the granzyme entered the nucleus of the targets (Figure 6.3; Tables 6.1 and 6.2).

To test the hypothesis that nuclear condensation was not mediated by caspase-3, we assessed nuclear morphology of target cells labeled with the DNA binding dye, DAPI. Figure 6.4 shows cell that have been treated with the caspase-3 inhibitor followed by granzyme B and Ad2 or perforin. Although these cells did not display TUNEL positivity (Figure 6.3) many showed signs of condensed nuclei. These results demonstrate that nuclear condensation in granzyme B mediated apoptosis is driven through some activity other than caspase-3.

It would appear that caspase-3 is required for DNA fragmentation in granzyme B treated cells but is dispensable for nuclear translocation of granzyme B and condensed nuclear morphology. These data support the notion that granzyme B-dependent DNA fragmentation is mediated through activation of caspase-3 or another caspase and that the granzyme induces nuclear condensation indirectly, likely through the activation of the laminase, caspase-6.

Figure 6.3 Inhibition of caspase-3 (CPP32) inhibits DNA fragmentation but not nuclear translocation of granzyme B. Jurkat targets were treated with *z*-DEVD-fmk for 30 minutes prior to incubation with granzyme B for 60 minutes and perforin (panel a) or Ad2 (panel b) for an additional 60 minutes (all at 37°C). Cells were labeled with α-granzyme B and Texas Red™-conjugated secondary antibody and TUNEL with dUTP-FITC. A lack of green label in these cells is indicative of the absence of DNA fragmentation. However, there is a significant nuclear accumulation of granzyme B. scale bar is 10 μm.

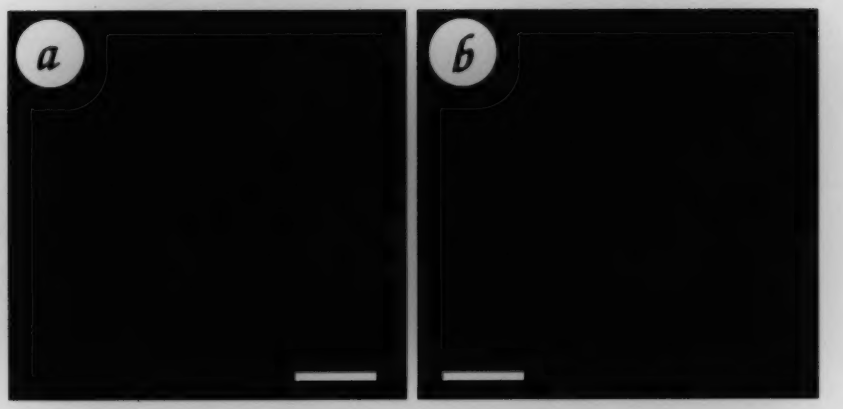
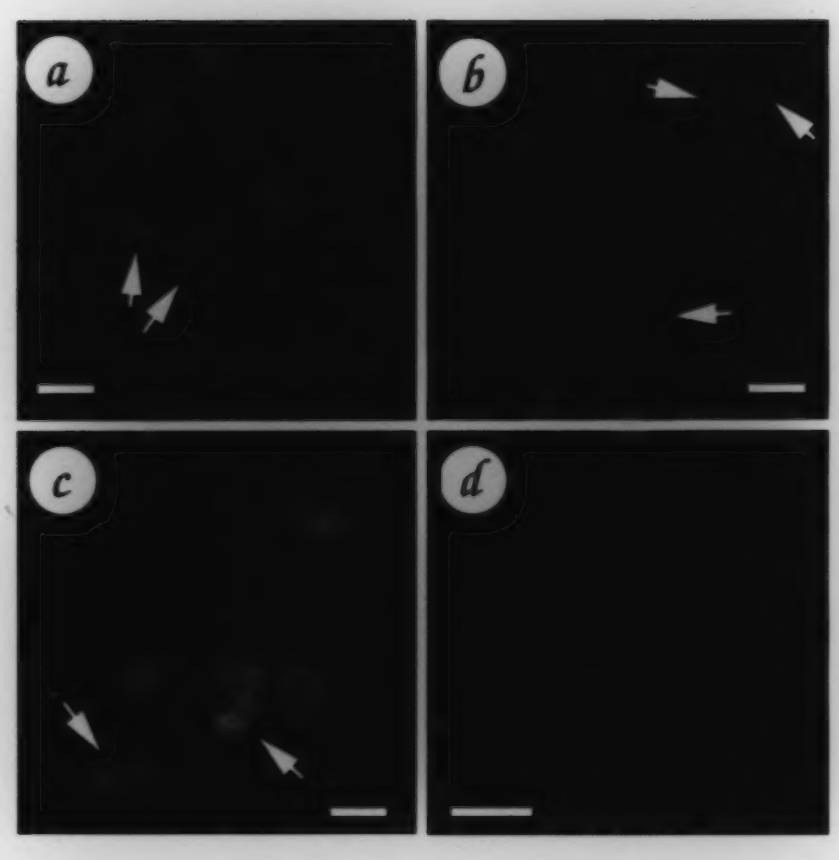

а
б

Figure 6.4 Nuclear condensation occurs in the absence of DNA fragmentation: inhibition of CPP32 inhibits DNA fragmentation but not nuclear condensation. Panels (a) and (b) show Jurkats that were incubated for 60 minutes to allow uptake of granzyme B. Cells were washed and incubated in the CPP32 inhibitor, z-DEVD-fmk, followed by treatment with sublytic levels of perforin (panel a) and Ad2 (panel b). Cells were stained with DAPI for nuclear morphology. COS M5 expressing granzyme B and granzyme B[S183A] treated with sublytic levels of perforin, stained with DAPI, are shown in panels (c) and (d) respectively. Examples of condensed nuclei of apoptotic cells are highlighted by arrows. Scale bar is 10 μm.


а
б
с
d

Nuclear translocation as a cause or consequence of cell death

Catalytically active granzyme B is required for death, but the inactive mutant still accumulated in the nucleus after perforin/Ad2 treatment. Nuclear translocation of the enzyme cannot, therefore, be explained as a result of nuclear membrane leakiness during apoptosis. Indeed, as indicated in Table 6.1, granzyme B appeared in the nucleus prior to DNA fragmentation suggesting that this movement may be the cause of apoptosis. In a complementary approach to address the role of nuclear translocation, granzyme B containing Jurkat target cells were stimulated to undergo apoptosis by a granule-independent mechanism. To achieve this we treated Jurkat cells, loaded with granzyme B, with antibody to the surface molecule FAS (Apo-1). Cells displayed PS externalization as measured by binding of annexin V-FITC and FACS analysis. These cells were also immunolabeled for granzyme B and fluorescent TUNEL and viewed by CLSM. At two hours approximately 25% of cells were TUNEL positive. Although these cells displayed gross breakdown of nuclear morphology as well as DNA fragmentation, the labeling of granzyme B was not co-localized with TUNEL label as was observed in cells undergoing apoptosis by stimulation with granzyme B and perforin or Ad2 (Figure 6.1). We therefore conclude that the appearance of granzyme B in the nucleus in granzyme B/perforin-mediated killing is not merely the consequence of a generalized disruption of the nuclear membrane as the cell undergoes apoptosis.

Granzyme B does not directly cleave lamin A

Proteolytic degradation of the nuclear lamina is an integral step in the dismantling of an apoptotic cell. Although caspase-6 (Mch2α) has recently been shown to be responsible for laminase activity in HeLa nuclei, there remained the possibility that cleavage of lamin A was due to granzyme B activity in granule-mediated killing of targets. To test the potential proteolytic activity of granzyme B toward lamin

A, we used lamin A that was transcribed and translation *in vitro* from the a full length open reading frame cloned into the expression vector Bluescript pSK+. ^{35}S-Met lamin A was incubated for 30 minutes in the presence of granzyme B. Caspase-3, which was generated in the same manner to avoid the possibility of inhibitors being present in the translation mixture, was used as a positive control for granzyme B activity. Figure 6.6 shows that granzyme B, in amounts sufficient to cleave caspase-3, does not cleave lamin A.

DISCUSSION

Nuclear Translocation of Granzyme B is Associated with Apoptotic Cell Death

A remarkable observation was that granzyme B could be rapidly translocated to the nucleus of the target cell but only if released from its endosomal pathway by a membraneolytic event. To determine whether nuclear translocation was a cause or a consequence of cell death was addressed in two ways. It was shown that the inactive granzyme B could undergo translocation in healthy cells if an endosomalytic agent was used. Further, induction of apoptosis through another pathway (anti-Apo-1 mAb) demonstrated that nuclear translocation of granzyme B does not occur. Together, the results suggest that in order to enter the nucleus, granzyme B must be released to the cytosol. Further, the absence of translocation implies that the granzyme remains limited to an endosomal pathway and that apoptosis is not associated with a generalized disruption of intracellular vesicular pathways.

As a granzyme-independent apoptotic stimulus, we used anti-FAS IgM to signal through the surface molecule, Fas/Apo-1. Onset of DNA fragmentation and PS externalization was observed as previously described (Martin et al., 1995, 1996). However, we did not observe any granzyme B co-localizing with the TUNEL label. The labeling pattern of granzyme B in anti-Fas stimulated cells was markedly different

Figure 6.5 Granzyme B does not directly cleave lamin A. Radiolabeled Lamin A was produced by coupled *in vitro* transcription and translation. Caspase-3 was produced in the same manner to serve as a positive control. Translation products were incubated in the noted concentration of granzyme B for 60 minutes at 37°C and run on a 10% polyacrylamide gel, fixed in enhancing solution and exposed to x-ray film.

negative
granzyme B (0.2ug)
granzyme B (1.0ug)
granzyme B (2.0ug)
granzyme B (10ug)
granzyme B (1.0ug)
negative
lamin
CPP32
(caspase-3)
p17

from the nuclear labeling observed in perforin or Ad2 stimulated targets. This suggests that release of granzyme B is not occurring in the same manner as with perforin or Ad2. Since granule- and Fas-mediated cytotoxicity are believed to converge at a point downstream of granzyme B, it appears that nuclear translocation of granzyme is not simply from breakdown of intracellular structure and/or due to loss of nuclear membrane integrity.

The mechanism that results in nuclear localization of granzyme B is unclear. Granzyme B does not contain a linear or bipartite nuclear localization signal (Görlich and Mattaj, 1995; Davis, 1995), nor is the granzyme involved in known procesess of nuclear import (Jans, 1995). There remains a distinct possibility that translocation is facilitated by a carrier protein (see Davis, 1995). Although nuclear substrates of granzyme B have not been identified, increasing evidence suggests they exist (Pinkoski et al., 1996; Trapani et al., 1996). We have previously demonstrated by protease-linked immunoelectron microscopy that granzyme B binds to a protein associated with heterochromatin and the nucleolus (Pinkoski et al., 1996) and a recent study demonstrated that the earliest events in DNA fragmentation in apoptosis are associated with heterochromatin in vitro in TNF-alpha treated L929 fibroblasts and in vivo in the developing chick embryo (Sanders and Wride, 1996).

Granzyme B is involved in many facets of apoptotic killing

Activation of caspase-3 by granzyme B has been shown to be required for granule-mediated apoptosis (Darmon et al., 1995). To investigate the involvement of caspase-3, we used an irreversible substrate analog inhibitor, z-DEVD-fmk, which inhibits CPP32-like caspases. The localization of caspase-3 in the cytosol and the necessity of caspase-3 to produce DNA fragmentation suggests that granzyme B cleaves caspase-3 in the cytoplasm prior to nuclear translocation of both proteinases where caspase-3 is responsible for cleaving PARP (Nicholson et al., 1995; Tewari et

al., 1995). Jurkat cells that are not undergoing apoptosis display a cytoplasmic labeling pattern for caspase-3 (Pinkoski and Bleackley, unpublished observations). If pro-caspase-3 resides in the cytoplasm, then granzyme B activates caspase-3 in the cytosol before transport of the two proteinases to the nucleus. It is important to note that z-DEVD-fmk did not adversely affect the accumulation of granzyme B in the nucleus. Therefore, caspase-3 activity is not required for the nuclear translocation of granzyme B.

Granzyme B rendered inactive with DCI was observed inside the targets and, after addition of perforin or Ad2, was translocated to the nucleus. DNA fragmentation, however, was not observed in these cells. Similarly, perforin and Ad2 both stimulated nuclear translocation of catalytically inactive granzyme B[S183A] in COS cells without DNA fragmentation. However, when catalytically active protease was used in these two models for granzyme B delivery, DNA fragmentation was always observed. From these results we concluded that enzymatic activity of granzyme B, although not required for binding and internalization or for translocation to the nucleus, is essential for cell death.

When cells are treated with z-DEVD-fmk there is an abrogation of DNA fragmentation. Strikingly, nuclear condensation is still observed in these cells. This suggests that, although granzyme B-mediated DNA fragmentation is mediated through caspase-3, granzyme B acts on as yet unidentified nuclear substrates to cause nuclear condensation. This also indicates that nuclear condensation and DNA fragmentation are two distinct steps in the apoptotic program.

Our results indicate that the appearance of granzyme B in the nucleus precedes the appearance of apoptotic nuclear events and thus suggests that it is an important event. Although at the present time we cannot prove that translocation of granzyme B to the nucleus is a key step in the induction of apoptosis, we have clearly established that it occurs and is not merely a consequence of cell death.

Granzyme B and lamin proteolysis

Lamin proteolysis is believed to be one of the key steps in dismantling of the apoptotic nucleus. Since we had observed nuclear condensation in the absence of DNA fragmentation, it was apparent that lamin proteolysis was occurring in the absence of active caspase-3. We used *in vitro* transcribed and translated lamin A to test lamin A as a possible substrate for granzyme B. Using amounts of granzyme sufficient to cleave caspase-3, we observed no cleavage of lamin A. Since granzyme B did not cleave lamin *in vitro*, it is likely that granzyme B triggers nuclear condensation through the activation of other proteases, likely caspases, that are not inhibitable by the caspase-3 inhibitor *z*-DEVD-fmk.

Granzyme B has been shown to cleave and activate the laminase caspase-6. Therefore, granzyme B activation of both caspase-3 and caspase-6 would result in DNA fragmentation and nuclear condensation, but would be carried out by independent means downstream of granzyme B. The results presented in this chapter, taken together with the observation of a nuclear granzyme B binding protein, suggest an unknown nuclear activity for granzyme B.

In Chapter III we established that granzyme B recognizes a nuclear protein associated with heterochromatin. Here we add further evidence to suggest a nuclear role for granzyme B, by demonstrating that granzyme B is rapidly translocated to the nucleus upon induction of apoptosis. Nuclear translocation of granzyme is not dependent on enzymatic activity. We also showed that DNA fragmentation is dependent on active granzyme B and is mediated through activation of caspase-3, whereas nuclear condensation mediated by granzyme B occurs independently of caspase-3.

CHAPTER VII

DISCUSSION

CHAPTER VII

DISCUSSION

The period during which these studies were conducted represents a time of unprecedented growth in the field of apoptosis. Apoptosis and programmed cell death were characterized by morphological descriptions and biochemical hallmarks with little information about how these processes are governed. There has since been the discovery of a protease cascade that is instrumental in executing the death program.

Similarly, the accepted model of CTL mediated cytotoxicity has also evolved significantly during this time. Perforin was believed to form channels in the plasma membrane large enough to permit passage of granzymes into the target. The effect of granzymes was believed to be of a destructive nature. That is, the proteases got into the cytosol and degraded target cell proteins, resulting in cell death.

However, much of what was accepted as paradigm has been discarded for a more complex model of a tightly regulated system in which granzyme B gains entry into the target cell and activates a protease cascade that serves as the death program. Rather than merely degrading target cell proteins, granzyme B cleaves and activates the caspases. The caspases in turn stimulate further proteases and nucleases resulting in a systematic dismantling of the cell.

The studies presented herein outline new evidence that has forced us to re-examine the regulation of granzyme B entry and trafficking in the target cell. We have found that granzyme B appears to be taken up by the target by receptor mediated endocytosis and then subjected to further trafficking and compartmentalization. These findings have also forced us to look for a physiologically relevant role for perforin.

Since perforin is not needed for granzyme B to enter the target, we are faced with two important questions: how is granzyme B getting into the target? and what is perforin doing?

Trafficking of granzyme B

We addressed the first question by investigating the possible routes of entry for soluble granzyme B: pinocytosis and receptor mediated endocytosis. Our first clue was provided by Froelich et al. (1996a) who demonstrated the presence of saturable binding sites for granzyme B on the surface of target cells. This study, however, failed to demonstrate autonomous entry of granzyme B and simply proposed that granzyme B remained bound to the outside of the cell until perforin was added to facilitate uptake according to the macromolecular channel model. Using immunocytochemical labeling to detect granzyme B and visualization by CLSM, we were able to ascertain that granzyme B did, in fact, enter the target cell in the absence of perforin. There was, however, no sign of cell death until perforin was added to the granzyme B-containing cells.

Through a series of temperature inhibition studies, we also acquired data to support the granzyme receptor theory and we demonstrated that granzyme B not only binds to a surface receptor, which was the conclusion of Froelich's group, but we also showed that granzyme B enters the target cell autonomously and is transported into an endocytic vesicle.

Entry of granzyme B is not a proteolytic event so it will be worthwhile to ascertain the region of granzyme B necessary for interaction with the putative granzyme receptor. This could be performed easily using radiolabeled polypeptide fragments corresponding to granzyme B. Regions of the protein that are accessible to the solvent would be a logical place to start. Armed with the knowledge of the region of granzyme B necessary for receptor-granzyme interactions, one should be able to isolate and

identify the granzyme B receptor by affinity chromatography techniques or far-western screening of an expression library.

Granzyme B appears to be subjected to more than one level of control in the target cell, since the protease can gain entry into the cell but cannot gain access to substrates necessary for induction of the death program. We have shown that intracellular trafficking of granzyme B is dependent on the glycosylation state of the enzyme. This should provide us with valuable clues toward delineating the path followed by granzyme B inside the target.

The mannose phosphate receptor in granzyme mediated cytotoxicity

MPR, through interactions with M6P moieties, is known to mediate entry of M6P containing proteins. Competition with soluble M6P did not decrease the number of cells taking up granzyme B. Although M6P did not appear to be adversely affecting entry of granzyme B, it did partially inhibit killing. This result appears to be significant since unphosphorylated mannose did not have any inhibitory effect on killing. The contribution of carbohydrate moieties on granzyme play some critical role in target cell trafficking of granzyme B leading to death, since the unglycosylated form of granzyme B failed to induce any aspects of apoptotic death assessed. Perhaps there is some contribution from the core carbohydrate that is partially dependent on M6P and independent of mannose.

There remains the possibility that there is more than one means of entry, or that there is more than one contributing factor for efficient entry. In this way, M6P would contribute to uptake, but would not be wholly responsible. To address this possibility inhibition studies will have to be performed with permutations of known and suspected inhibitors to assess the contribution of each.

Although the MPR appears to be the primary means by which granzyme B is directed to the secretory granule in CTL, it is most likely not the only one. Patients

with I-cell disease cannot process high mannose glycosylation of proteins and therefore granzyme B acquires a complex glycan pattern of glycosylation and is not transported to the secretory granule by the MPR. However, there remains significant activity of lymphocytes to perform regulated secretion in order to kill targets (Griffiths and Isaaz, 1993). Since a compensatory pathway exists for transporting granzyme B to the secretory granule in the CTL, it seems a distinct possibility that a similar pathway exists in the target cell.

A new role for perforin?

If perforin is not needed to get granzyme B into the cell, then what is its role? Perforin is undeniably an important cytolytic effector molecule. However, data presented here has forced us to re-assess the physiologically relevant activity of perforin in CTL mediated killing. Since the macromolecular conduit for granzyme transport is not necessary, it is likely that perforin is acting downstream of granzyme entry into the target. One of the earlier functions of perforin is described as a non-selective ion channel (Podack et al., 1985; Young et al., 1986c; Ojciuset al., 1991). Perhaps perforin is acting by stimulating ion flux in the target cell. Imbalance of the ion gradients in the cell could result in disruption of intracellular vesicles.

Interestingly, K^+ efflux has been observed during apoptosis. Perforin also shares a physical similarity with voltage gated K^+ ion channels. Voltage gated channels tend to exist as tetramers (Cattarall, 1995), the same subunit proposed as the limiting number in a polyperforin channel (Rochel and Cowan, 1996). Upset of ion gradients leading to a disruption of endosomes may provide a means to discharge the granzyme into the cytosol without requiring a drop in endosomal pH. One might envisage a system in which granzyme B is sequestered in a cytoplasmic vesicle and perforin disrupts the gradients that are necessary to maintain these vesicles. This would result in the release of granzyme B from its compartment and bring it in contact with its

substrates. Since granzyme B and perforin are almost certainly delivered simultaneously *in vivo*, accumulation of granzyme B would never occur; i.e. perforin may upset vesicular integrity while granzyme B is in transit prior to compartmentalization.

Since perforin is not needed at the plasma membrane for granzyme entry, it is possible that perforin is also subjected to trafficking within the target cell. By insertion into the membrane of intracellular organelles, a polyperforin ion channel might cause a disruption in endosomes resulting in release of endosomal contents. Disruption of a key organelle in this manner could conceivably have a profound, amplifiable effect on the integrity of many cytoplasmic organelles.

Nuclear role of granzyme B

We clearly established that granzyme B recognizes a heterochromatin associated protein. This observation, taken with nuclear translocation of granzyme B at the onset of DNA fragmentation, we are led to believe that there is some nuclear function for granzyme B during apoptosis. It is possible that activation of caspase-6 occurs in the nucleus prior to breakdown of the nuclear lamina. To investigate this possibility we would have to inhibit nuclear transport of granzyme B and assess the effect on killing. Since the mechanism by which granzyme B is transported to the nucleus is not known, these will be difficult experiments to perform using non-specific inhibitors of nuclear transport that would also inhibit nuclear transport of prospective substrates.

The 80 kDa protein was shown to be immunoprecipitable and should therefore be the target of an intensive effort for its identification. Attempts to purify heterochromatin associated proteins proved unsuccessful, but it should be possible to purify enough protein by immunoprecipitation for N-terminal microsequencing.

Nucleolar and heterochromatin associated proteins as potential substrates

One of the hallmarks of apoptosis is nuclear disintegration (Kerr et al., 1972) followed by DNA fragmentation and chromatin condensation (Duke et al., 1983). Based on the data presented here, a hypothesis emerges in which granzyme B may cleave a substrate that is responsible for maintaining the condensation of heterochromatin. Such an event may then result in the exposure of a DNase sensitive site or motif that would otherwise be sequestered. In this model granzyme B would bring about the destruction of a target cell by rendering the target cell sensitive to destructive machinery that is constituitively present and active in the target cell.

The nucleolar labeling pattern includes a specific association with the perinucleolar region and nuclear envelope. There is one nuclear heterochromatin-associated protein of 80 kDa that is part of the centromere binding framework, CENP-B, which fulfilled the two physical criteria we desired. CENP-B has an N-terminal DNA binding domain and a C-terminal homodimerization domain. It is believed to be responsible for packaging of DNA into the condensed form that we observe as heterochromatin. One might envisage that cleavage of this molecule would result in decondensation of the heterochromatin which would result in exposure of the DNA to existing endonucleases.

In fact CENP-B contains a potential granzyme B cleavage site that is conserved in all species identified. An LETD sequence has only a minor conservative substitution of Leu for Ile at P4. Proteolytic cleavage at this site in CENP-B would result in dissociation of the dimerization domains.

Future Directions

If the role of perforin is not to facilitate entry of granzyme B then what is it doing? Clues to the physiological activity may be provided by comparative studies with

perforin and Ad2. Obviously, these two agents can induce a similar response in the target cell and so must share some common biological feature(s). Finding the similarity between perforin and Ad2 will provide us with valuable information regarding a biologically relevant activity of perforin.

Since perforin is not required to act at the plasma membrane to mediate granzyme entry, it is now questionable as to whether or not this is the actual site of action for perforin. The N-terminal 34 residues of perforin have been shown to possess the transmembrane and polymerization domain and the ion channel activity. Perhaps there is some targeting motif contained within the remainder of the protein. It should be technically straightforward to use confocal microscopy to analyze immunolabeled perforin in target cells. It is important that sub-lytic doses of perforin be used in these experiments to be physiologically relevant. If perforin is acting at some intracellular site, we could also determine the site by immunolocalization. Knowing the site of action of perforin would be invaluable in determining the nature of its biological activity.

Granzyme B has been shown to accumulate in the nucleus and binds to a nuclear protein associated with heterochromatin. However, a nuclear activity for granzyme B is still unknown. What then, is granzyme B doing in the nucleus? A stringent search for nuclear substrates and further characterization of the 80 kDa protein previously described will be necessary.

The effects of nuclear translocation of granzyme B have yet to be conclusively determined. It would be interesting to examine the extent of granzyme B effects if nuclear transport is inhibited. Since caspase cleavage by granzyme B is presumably in the cytoplasm, this might be a reasonable means to distinguish between nuclear and cytoplasmic effects of granzyme B. Of course, if we could selectively inhibit nuclear transport of granzyme B and not caspase-3 or caspase-6, it would allow us to further

dissect the nuclear effects of the caspases from those directly attributable to granzyme B.

We have shown that granzyme B binds to at least four unidentified proteins in the cell. While it will be interesting to know the identity of the three potential substrates, it will also be interesting to know the identity of the 69 kDa protein that is recognized by both the active granzyme and the zymogen. In light of our evidence supporting the notion of receptor mediated endocytosis and that entry, trafficking and nuclear transport are not dependent on protease activity, the 69 kDa protein may be involved in the transport of granzyme B within the target cell.

Now that many of the cellular processes in cell death are being identified, there is an effort to determine the order in which these events occur. This helps to determine which effects cause morphological alterations and which are merely coincidental. If we can place specific trafficking events and proteolytic activities of granzyme B within the context of other biochemical features of target cell apoptosis, we may be able to better define the underlying mechanisms. Early studies in CTL and NK cell killing focused on changes in pH and ion flux. There is now a resurgence in this area of study, including observations of changes in the permeability transition in the mitochondria. Since both of the these processes may cause disruptions in pH gradients and the vesicles maintained thereby, it will be interesting to know the contribution of these biochemical changes in the apoptotic target cell. Do either the Na^+/H^+ or K^+/H^+ gradients and the membrane potential of the outer mitochondrial leaflet play a role in controlling target cell death in this system?

Conclusions

The data presented in this thesis, as well as additional studies in this rapidly moving field, have led us to re-examine the mechanism of granzyme-perforin mediated apoptosis. We have data to support a model that can be summarized as follows.

Granzyme B binds to a surface receptor on target cells, the proteinase is internalized by receptor mediated endocytosis and is subsequently sequestered from degradation in an early and/or recycling endosome. At the time of perforin stimulation, presumably concurrent *in vivo*, granzyme B is released into the cytosol where the primary caspase substrates are encountered. After initiation of the caspase-mediated death pathway through cytosolic events, granzyme B is translocated to the nucleus whereupon it binds to nuclear substrates, as yet unidentified. One of these potential nuclear substrates is associated with heterochromatin and the nucleolus.

The work presented here encompasses novel findings in the mechanism of granzyme B action inside the target cell. Perhaps even more compelling and undoubtedly more confounding are some of the issues raised as a result of these findings. We are being forced to re-assess the role of perforin in granule mediated killing. The macromolecular granzyme conduit model of perforin is now unlikely and we must search more earnestly for the physiological role of perforin in granule mediated killing.

CHAPTER VIII

BIBLIOGRAPHY

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CHAPTER IX

VITA

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Personal

Date of Birth: June 13, 1966

Place of Birth: Edmonton, Alberta, Canada

Address: 333 Kline Crescent
Edmonton, Alberta
Canada, T6L 6K8

Telephone: (403) 468-9222 (home)
(403) 492-4552 (lab)
(403) 492-0886 (fax)

E-mail: mjp1@gpu.srv.ualberta.ca

Academic

09/94 to present: Doctor of Philosophy
Department of Biochemistry
University of Alberta
Edmonton, Alberta, Canada
Thesis Title (proposed): The Role of Granzyme B in Target Cell Apoptosis
Proposed date of completion: 08/97.

09/88 to 08/91: Master of Science
Department of Pathology
Cancer Research Laboratories
Queen's University
Kingston, Ontario, Canada
Thesis Title: Identification of a Resistance Associated Antigen in a Multidrug Resistant Small cell Carcinoma Cell Line.
Degree Awarded: M.Sc. (Pathology) 08/91.

09/84 to 04/88: Bachelor of Science
Department of Biochemistry
University of Alberta
Edmonton, Alberta, Canada
Degree Awarded: B.Sc. (Biochemistry) 04/88.

Invited Lectures and Presentations

La Jolla Institute for Allergy and Immunology, San Diego, CA, June 12, 1997. Granzyme B and perforin killing re-visited.

Canadian Society for Immunology Spring Symposium, Lake Louise, Alberta. March, 1997. Workshop seminar: Cytotoxic T Lymphocyte Killing: Autonomous Entry of Granzyme B into Endosomes Followed by Perforin Dependent Release and Apoptosis.

Cancer Biology Research Group, University of Calgary, Calgary, Alberta, March 6, 1997. "Mechanisms of cell mediated cytotoxicity: paradigm lost?"

University of Toronto Department of Pathology Mini Symposium. Toronto, Ontario, November 29, 1990. Investigation of resistance associated antigens in a multidrug resistant small cell carcinoma cell line.

Awards and Honours

Canadian Society for Immunology, Spring Symposium, 1997, Student Poster Award

Canadian Society for Immunology, Cedarlane Award, First Place in Student Travel Awards for the Spring Symposium, 1997

Keystone Symposium on Apoptosis and Programmed Cell Death, 1997, Student Poster Award

J. Gordon Kaplan Graduate Student Award, 1997

University of Alberta, Department of Biochemistry, Tuition Scholarship, 09/96

Alberta Heritage Foundation for Medical Research, Full Time Studentship, 09/94 to present

Cancer Research Society of Canada, Pre-doctoral Fellowship 07/89 to 06/91

Alberta Heritage Trust Fund, A.C. Rutherford Scholarship, 1984

Knights of Columbus Scholarship for Post-Secondary Education, 1984

Publications

Pinkoski, M.J., Heibein, J., Hobman, M., Caputo, A., Froelich, C.J., and Bleackley, R.C. Target cell trafficking events required for granzyme B mediated apoptosis. *Manuscript in preparation*

Darmon, A.J., **Pinkoski, M.J.**, and Bleackley, R.C. (1997). Granzyme B mediated cytotoxicity. *In*

Pinkoski, M.J., Caputo, A., Hobman, M., Tomaselli, K.J., Feng, L., Seth, P., Froelich, C.J., and Bleackley, R.C. (1997) Cytotoxic T Lymphocyte Killing: Autonomous Entry of Granzyme B into Endosomes Followed by Perforin Dependent Release and Apoptosis. *Submitted.*

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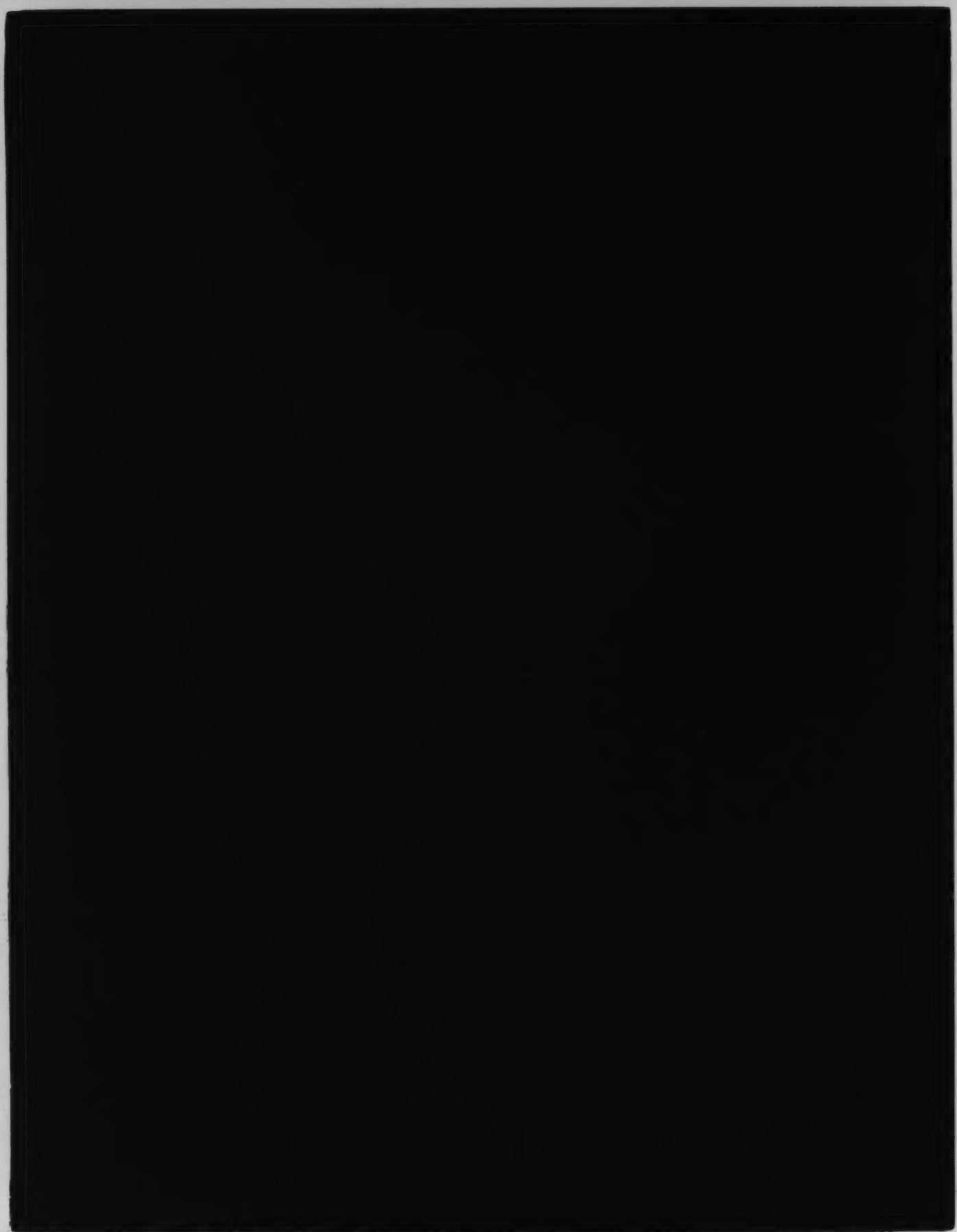
Prendergast, J.A., **M. Pinkoski**, A. Wolfenden and R.C. Bleackley (1991). Structure and evolution of the cytotoxic cell proteinase genes CCP3, CCP4 and CCP5. *J. Mol. Biol.* 220: 867-875.

Abstracts

Pinkoski, M.J., Caputo, A., Froelich, C.J., and R.C. Bleackley. Cytotoxic T Lymphocyte Killing: Autonomous Entry of Granzyme B into Endosomes Followed by Perforin Dependent Release and Apoptosis. Canadian Society for Immunology Spring Symposium, Lake Louise, Alberta. March, 1997.

Pinkoski, M.J., Froelich C.J., and R.C. Bleackley. Nuclear Translocation of Granzyme B Coincides with Cell Death Induced by Cytotoxic T Lymphocytes. Keystone Symposium on Molecular and Cellular Biology: Apoptosis. Tamarron, Colorado, February 18-23, 1997.

Pinkoski, M.J. and R.C. Bleackley. Granzyme B binds a nuclear protein of 80 kDa and cytoplasmic proteins of 50, 69 & 94 kDa in target cells. ASBMB/ASIP/AAI Joint Meeting (American Association of Immunologists). New Orleans, LA, June 1-7, 1996

Pinkoski, M.J. and R.C. Bleackley. Granzyme B binds a nuclear protein of 80 kDa and a cytoplasmic of 94 kDa in EL4 target cells. Keystone Symposium on Molecular and Cellular Biology: Apoptosis. Tamarron, Colorado, March 5-11, 1995.

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